# ADULT STEM CELLS FOR REGENERATIVE MEDICINE: FROM CELL FATE TO CLINICAL APPLICATIONS

EDITED BY: Sudjit Luanpitpong, Pakpoom Kheolamai and Jingting Li
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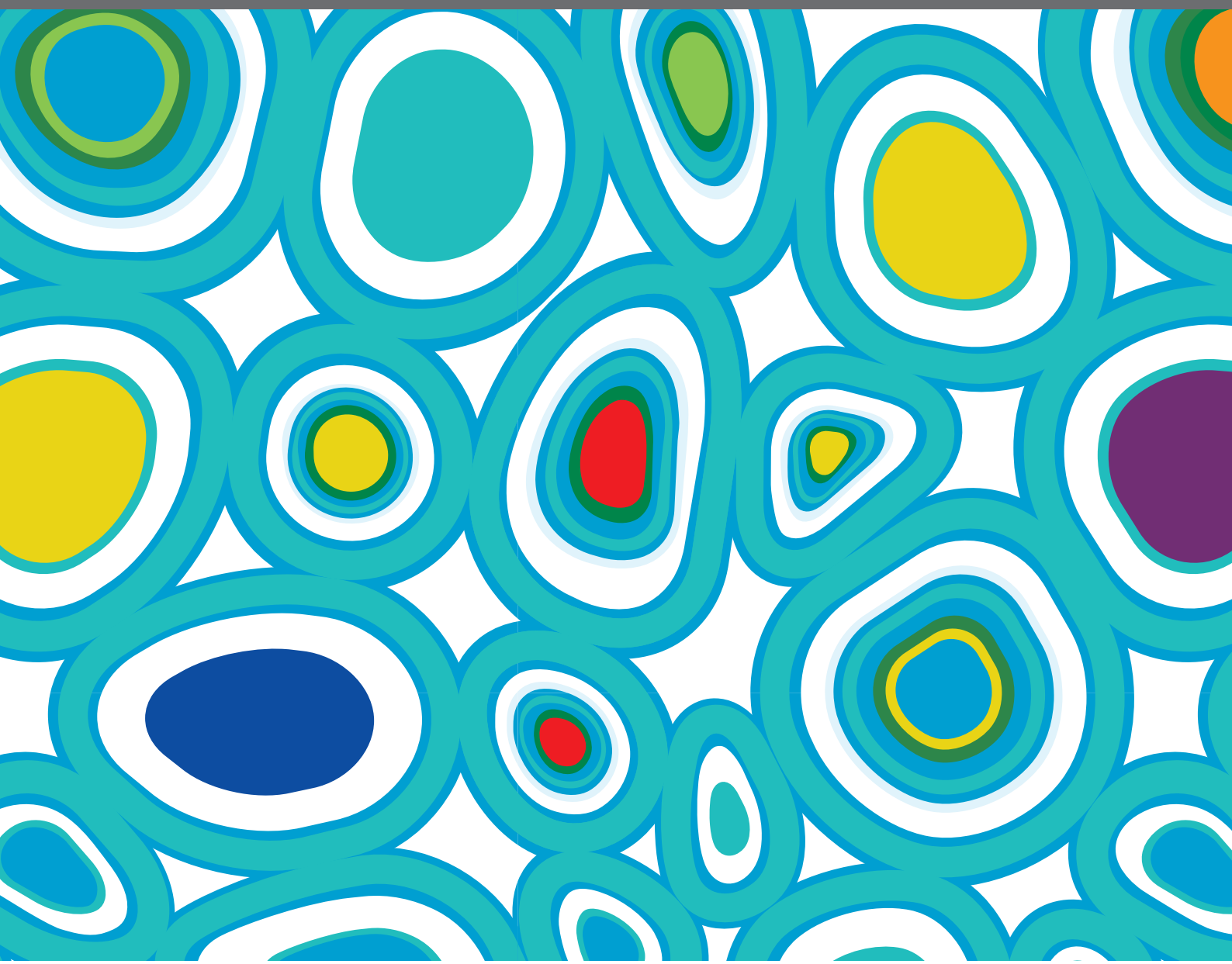

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# ADULT STEM CELLS FOR REGENERATIVE MEDICINE: FROM CELL FATE TO CLINICAL APPLICATIONS

Topic Editors:
**Sudjit Luanpitpong,** Mahidol University, Thailand
**Pakpoom Kheolamai,** Thammasat University, Thailand
**Jingting Li,** Sun Yat-sen University, China

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EDITED AND REVIEWED BY
Valerie Kouskoff,
The University of Manchester,
United Kingdom

*CORRESPONDENCE
Sudjit Luanpitpong,
suidjit@gmail.com
Pakpoom Kheolamai,
pkheolamai@me.com

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# Editorial: Adult stem cells for regenerative medicine: From cell fate to clinical applications

Jingting Li[1,2,3,4], Sudjit Luanpitpong[5]* and Pakpoom Kheolamai[6]*

[1]Institute of Precision Medicine, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China, [2]Department of Burns, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China, [3]Department of Dermatology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China, [4]Department of Neurosurgery, Institute of Precision Medicine, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China, [5]Siriraj Center of Excellence for Stem Cell Research, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, [6]Center of Excellence in Stem Cell Research and Innovation, Faculty of Medicine, Thammasat University, Bangkok, Thailand

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**stem cells, regenerative medicine, mesenchymal stem cells, bone marrow mesenchymal stem cells, dental pulp stem cells, limbal stem cells, epidermal stem cells, skeletal stem cells**

Editorial on the Research Topic
Editorials: Adult stem cells for regenerative medicine: From cell fate to clinical applications

Adult stem cells have emerged as a key player in cell-based therapy in regenerative medicine. With the multilineage differentiation capability and ability to secrete various potent bioactive molecules, hematopoietic stem/progenitor cells (HSPCs) and mesenchymal stem cells (MSCs; also known as multipotent or mesenchymal stromal cells), in particular, have been considered a potential treatment for many debilitating diseases caused by injuries, inflammation, and age-related degeneration. The transplantation of these cells has been consistently shown to be safe; however, their therapeutic potential for many diseases and conditions varies between studies, attributable mainly to the differences in stem cell sources, donor selection, the isolation and expansion procedure, characterization criteria, number of transplanted cells, and disease severity. A major challenge when using adult stem cells for therapeutic applications includes the heterogeneous population of cells at the time of derivation and upon expansion in culture. The same type of adult stem cells derived from different tissues or even the same tissue from different donors exhibits distinct characteristics and biological properties, making the outcome of stem cell therapy difficult to be predicted. To improve the efficacy and reproducibility of stem cell therapy in regenerative medicine, a reliable characterization and a more thorough understanding of the mechanisms underlying the therapeutic effects of adult stem cells from various sources are critical. One of the well-established therapeutic mechanisms of HSPCs and MSCs involves the release of bioactive molecules,

which have been shown to reduce inflammation, increase cell viability, and enhance tissue regeneration in injured tissues. In this issue, Norte-Muñoz et al. demonstrated the prosurvival effect of cytokines released from bone marrow-derived MSCs (BM-MSCs) on mouse retinal ganglion cells that enhanced axonal regeneration of the mouse optic nerve. The therapeutic effects of BM-MSCs largely depend on the immunocompatibility between donors and recipients, and hence, the type of transplant in which the higher compatibility is generated is the better outcome. A report by Li et al. also demonstrated a novel therapeutic mechanism of rapamycin, a drug used to treat immune-mediated bone marrow failure, by showing that the drug induces BM-MSCs to release granulocyte–macrophage colony-stimulating factor (GM-CSF), which promotes the expansion of myeloid-lineage cells while suppressing the subsequent differentiation of those myeloid cells to granulocytes in a mouse model.

Since the early days of stem cell therapy, HSPCs, MSCs, and endothelial progenitor cells (EPCs) have been used to treat various chronic diseases, especially diabetes and vascular complications associated with diabetes, such as myocardial infarction, ischemic stroke, and peripheral vascular disease. Two review articles by Yu et al. and Khodayari et al. provide an overview of the use of various adult stem cells to treat diabetic foot ulcers and limb ischemia, which are the leading causes of morbidity in diabetic patients. However, the significant hurdle to stem cell therapy for diabetes and its related diseases is the low survival rate of the transplanted cells, especially in the ischemic and inflammatory microenvironments of the target tissues. In this regard, Zhang et al. summarized the various types of programmed cell death that compromise the survivability of MSCs after transplantation and the potential strategies to prevent them. This insight is essential for developing an intervention that increases the overall survival of transplanted MSCs, especially in patients whose tissues experience chronic ischemic and inflammatory conditions, such as diabetes and atherosclerosis. Reconstruction of the bone and muscle is one of the most promising clinical applications of adult stem cells. To make the reconstruction processes more efficient, the optimization of biocompatibility scaffolds and a better understanding of the molecular events accompanying the differentiation of MSCs toward the desirable lineages are critical. A report by Xin et al. showed the potential of CHNQD-00603, a modified natural product derived from an anamorphic fungus *Scopulariopsis* sp., in inducing osteogenic differentiation of BM-MSCs by enhancing their autophagy. A review article by Shen and Shi also highlights recent advances in identifying the molecular mechanisms underlying the interaction between MSCs and their microenvironment during osteogenic differentiation that could be used to improve the culture conditions to induce bone formation. In addition to the bone marrow and their BM-MSCs, the periosteum has been shown to play essential roles in bone formation in physiological and pathological conditions. A review article by Zhang et al. summarized current knowledge on lesser-known periosteum-derived periosteal skeletal stem cells (P-SSCs) and their role in bone formation after injury. These cells might be used as an alternative source of MSCs for bone reconstruction in addition to BM-MSCs, which are very limited in supply. Another source of MSCs that shows great potential in tissue engineering is dental pulp stem cells (DPSCs), which can be used to regenerate a typically irreplaceable dental pulp in endodontic treatment. In their review article, Kwack and Lee summarized the recent updates and limitations in using DPSCs to regenerate dental pulp in the clinics.

Although the use of stem cells to generate skin and corneal tissue pieces for transplantation has been established for quite some time, the reconstruction of these tissues requires a large number of cells, which remains a significant hurdle. Therefore, an effective strategy for expanding epidermal and corneal cells while preserving their differentiation potential is critical. A report by Sun et al. uncovered the signaling pathways and several target genes involved in the long-term expansion of human epidermal cells by single-cell RNA sequencing and predicted the possibility of cell–cell communication using CellChat, while a commentary article by Ji et al. provided helpful information about the optimized culture conditions, e.g., by adding small molecular compounds, for expanding mouse limbal stem cells, a corneal precursor. This information could be beneficial in discovering candidate targets to induce the regeneration of the injured skin and corneal tissues in patients. A research article by Shi et al. also reported the establishment of human urine-derived stem cells (USCs), which have extensive expansion capacity while maintaining their multilineage differentiation. This alternative source of MSCs that could be harvested from every patient using a non-invasive procedure could potentially be used for autologous stem cell transplantation.

Terminally differentiated skeletal muscle contains a population of stem cells called satellite cells, but its ability to regenerate a new muscle fiber after an injury is limited. A report by Kim et al. showed that a specific population in the pharyngeal muscle called fibroadipogenic progenitors (FAPs) induced the proliferation of satellite cells by releasing hepatocyte growth factor (HGF). Such a finding provides valuable information regarding the interaction between various cell populations in the muscle that might be used to enhance skeletal muscle regeneration by inducing the proliferation of its satellite cells.

We hope the novel discovery and insight provided by these research and review articles will enhance our knowledge about the great potential of various adult stem cells in regenerative medicine and bring us closer to real therapeutic interventions in the near future.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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# Neuroprotection and Axonal Regeneration Induced by Bone Marrow Mesenchymal Stromal Cells Depend on the Type of Transplant

*María Norte-Muñoz*[1], *Fernando Lucas-Ruiz*[1], *Alejandro Gallego-Ortega*[1], *David García-Bernal*[2], *Francisco J. Valiente-Soriano*[1], *Pedro de la Villa*[3], *Manuel Vidal-Sanz*[1] *and Marta Agudo-Barriuso*[1]*

[1]*Experimental Ophthalmology Group, Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca (IMIB-Arrixaca) and Universidad de Murcia, Murcia, Spain,* [2]*Hematopoietic Transplant and Cellular Therapy Unit, Molecular Biology and Immunology Department, Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca (IMIB-Arrixaca) and Biochemistry, Universidad de Murcia, Murcia, Spain,* [3]*Systems Biology Department, Faculty of Medicine, University of Alcalá, Alcalá de Henares, Spain*

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**Edited by:**
*Pakpoom Kheolamai, Thammasat University, Thailand*

**Reviewed by:**
*Hong Ouyang, Sun Yat-sen University, China*
*Johannes Boltze, University of Warwick, United Kingdom*

***Correspondence:**
*Marta Agudo-Barriuso*
*martabar@um.es*

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Mesenchymal stromal cell (MSC) therapy to treat neurodegenerative diseases has not been as successful as expected in some preclinical studies. Because preclinical research is so diverse, it is difficult to know whether the therapeutic outcome is due to the cell type, the type of transplant or the model of disease. Our aim here was to analyze the effect of the type of transplant on neuroprotection and axonal regeneration, so we tested MSCs from the same niche in the same model of neurodegeneration in the three transplantation settings: xenogeneic, syngeneic and allogeneic. For this, bone marrow mesenchymal stromal cells (BM-MSCs) isolated from healthy human volunteers or C57/BL6 mice were injected into the vitreous body of C57/BL6 mice (xenograft and syngraft) or BALB/c mice (allograft) right after optic nerve axotomy. As controls, vehicle matched groups were done. Retinal anatomy and function were analyzed *in vivo* by optical coherence tomography and electroretinogram, respectively. Survival of vision forming (Brn3a$^+$) and non-vision forming (melanopsin$^+$) retinal ganglion cells (RGCs) was assessed at 3, 5 and 90 days after the lesion. Regenerative axons were visualized by cholera toxin β anterograde transport. Our data show that grafted BM-MSCs did not integrate in the retina but formed a mesh on top of the ganglion cell layer. The xenotransplant caused retinal edema, detachment and folding, and a significant decrease of functionality compared to the murine transplants. RGC survival and axonal regeneration were significantly higher in the syngrafted retinas than in the other two groups or vehicle controls. Melanopsin$^+$RGCs, but not Brn3a$^+$RGCs, were also neuroprotected by the xenograft. In conclusion, the type of transplant has an impact on the therapeutic effect of BM-MSCs affecting not only neuronal survival but also the host tissue response. Our data indicate that syngrafts may be more beneficial than

**Abbreviations:** ATMP, advanced therapy medical product; BAF, blue autofluorescence filter; BM-MSC, bone marrow mesenchymal stromal cells; CTB, β subunit of the cholera toxin; DMEM, Dulbecco Modified Eagle Medium; ERG, electroretinography; FBS, fetal bovine serum; GFP, green fluorescent protein; hBM, human bone marrow; mBM, mouse bone marrow; MSC, mesenchymal stromal cells; OCT, optical coherence tomography; PBS, phosphate buffered saline; P/S, penicillin/streptomycin; R/T, room temperature; RGC, retinal ganglion cell; SD: standard deviation.

allografts and, interestingly, that the type of neuron that is rescued also plays a significant role in the successfulness of the cell therapy.

**Keywords: retinal ganglion cell, optic nerve crush, bone marrow mesenchymal stromal cells, syngraft, allograft, xenograft, neuroprotection, axonal regeneration**

## INTRODUCTION

The attractiveness of mesenchymal stromal cells (MSCs) as an advanced therapy medicinal product (ATMP) lays in their limited antigenicity, anti-inflammatory effects, immunomodulatory properties, and secretion of trophic factors (Le Blanc et al., 2003; Meirelles et al., 2009; Chaudhary et al., 2018; Mishra et al., 2020; Song et al., 2020; Wu et al., 2020; García-Bernal et al., 2021). Of similar importance, they are isolated quite easily from many niches of adult individuals (Kern et al., 2006; Hoogduijn et al., 2014; Valencia et al., 2016; Urrutia et al., 2019), avoiding the ethical problems of embryonic stem cells.

Stem cell therapy for neurodegenerative disorders has two main objectives, neuronal replacement (Coco-Martin et al., 2021) and neuroprotection (Millán-Rivero et al., 2018). In both cases, target reconnection is essential to restore function. Neuronal replacement is a very challenging task still unattainable for patients: circuitry is very complex, neurons are extremely specialized and highly diverse even within the same functional population (Rheaume et al., 2018; Tran et al., 2019). Ameliorating the course of neuronal death and the progression of the disease is a more attainable objective. Thus, although MSCs can differentiate into neurons and glia (Hernández et al., 2020), they are being trialled as neuroprotective ATMPs (Wright et al., 2011; Ng et al., 2014; Uccelli et al., 2019). Ongoing clinical trials involve a wide variety of conditions with different etiologies, such as spinal cord injuries, cerebral stroke, multiple sclerosis, Parkinson's, Alzheimer's, autism spectrum, glaucoma, or cerebellar ataxia. In these trials, majority of transplants are allogeneic (www.clinicaltrials.gov).

MSCs from different tissues are being assayed in many preclinical models of neurodegeneration (Zaverucha-do-Valle et al., 2011; Millán-Rivero et al., 2018; Mesentier-Louro et al., 2019; Ahani-Nahayati et al., 2021; da Silva-Junior et al., 2021; Figiel-Dabrowska et al., 2021; Li et al., 2021; Serrenho et al., 2021; Shabanizadeh et al., 2021). The origin of MSCs is crucial because their origin affects their plasticity, immunogenicity and stemness, which in turn will affect their response to *in vitro* amplification and to the host environment. The host species and tissue are also important, because the immune response is species (Zschaler et al., 2014; Webb et al., 2015) and tissue specific (Brown and Esterházy, 2021). Although MSCs have long been thought to be immunologically privileged (Aggarwal and Pittenger, 2005; English et al., 2010; Escacena et al., 2015; Gu et al., 2015), an increasing number of *in vitro* and *in vivo* studies have recently been described that MSCs induce both innate and adaptative host immune responses (Ankrum et al., 2014; Berglund et al., 2017), not only in xenotransplants (Jungwirth et al., 2018) but also in an allogeneic context (Dhingra et al., 2013; Oliveira et al., 2017). Thus, the balance between MSC secretome and MSC immunogenicity could be key for the MSC persistence in the host and in its mediated therapeutic response (Khan and Newsome, 2019).

Since most preclinical studies test human cells in rodents (xenograft) or cells from the same rodent species and strain (syngraft), and majority of clinical treatments are allogeneic, how can we reach translational conclusions based on preclinical experiments? To this, we must add that MSCs from different tissues are tested in different models of neurodegeneration, which are other variables that make it difficult to reach clear conclusions.

Here we purpose to study the effect of the transplant on neuroprotection and regeneration. For this we have grafted human and murine bone marrow-derived MSCs (BM-MSCs) into the vitreous body directly after optic nerve axotomy, a very well characterized model of neuronal degeneration (Sánchez-Migallón et al., 2018b; 2018a). BM-MSCs were chosen because in syngeneic transplants they have neuroprotective and neuroregenerative properties in models of CNS injury (Ankeny et al., 2004; Wright et al., 2011) including optic nerve lesions (Zaverucha-do-Valle et al., 2011; Mesentier-Louro et al., 2019).

## MATERIALS AND METHODS

### Animal Handling

All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Murcia (Murcia, Spain) and performed according to the guidelines of our Institution (approved protocols A13150201, A1320140704).

Two months old male mice (C57BL/6, BALB/c and C57BL/6-Tg (CAG-EGFP strains) were obtained from the breeding colony of the University of Murcia or purchased from Envigo (Barcelona, Spain) and The Jackson Laboratory (Bar Harbor, ME, United States), respectively. Animals were kept at the University of Murcia animal housing facilities in temperature and light controlled rooms (12 h light/dark cycles) with food and water administered *ad libitum*.

Optic nerve crush, intravitreal injections, OCT and ERG analyses were carried out under general anaesthesia administered intraperitoneally with a mixture of ketamine (60 mg/kg, Ketolar, Parke-Davies, S.L., Barcelona, Spain) and xylazine (10 mg/kg, Rompun, Bayer S.A., Barcelona, Spain). Analgesia was provided by subcutaneous administration of buprenorphine (0.1 mg/kg; Buprex, Buprenorphine 0.3 mg/ml; Schering-Plough, Madrid, Spain). During and after anaesthesia, eyes were covered with an ointment (Tobrex; Alcon S.A., Barcelona, Spain) to prevent corneal desiccation. Animals were sacrificed with an intraperitoneal injection of an overdose of

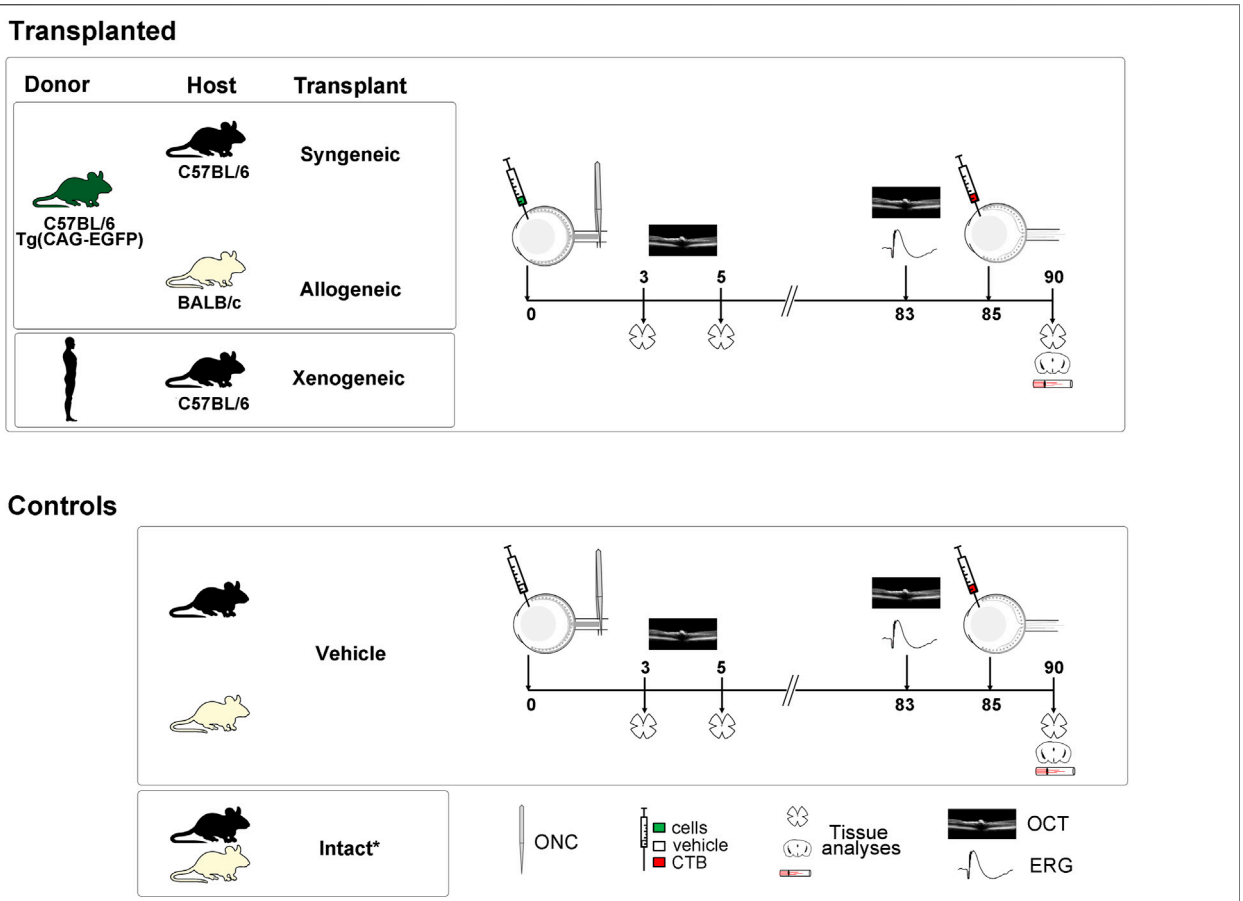


**FIGURE 1 |** Experimental design. *Controls: intact animals for anatomy and the right eyes for SD-OCT and ERG. Human and dark green mouse silhouettes: donors, Black and off-white mouse silhouettes: recipients and controls. Human and mouse drawings are modified from Vecteezy.com.

sodium pentobarbital (Dolethal, Vetoquinol; Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain).

## Experimental Design and Animal Groups

See **Figure 1**. Intact animals were used to assess the total number of RGCs because the undamaged contralateral retinas are not a suitable control (Lucas-Ruiz et al., 2019b). However, because the contralateral effect does not have a significant impact on the long term retinal thickness or functionality after axotomy (unpublished results), the right retinas of the experimental animals were used as control in the electroretinography (ERG) and spectral-domain optical coherence tomography (SD-OCT) analyses. This strategy allows reducing the number of animals because retinal thickness and functionality do decrease with age (Nadal-Nicolás et al., 2018).

## Isolation and Culture of Human and Mouse Bone Marrow Mesenchymal Stem Cells

Human bone marrow samples (hBM) were collected by iliac crest aspiration from 6 healthy volunteers without previous comorbidities (three men and three women, age 21–45 years old) after written informed consent and after the approval of the local Ethics Committee of the University Hospital Virgen de la Arrixaca (HUSA19/1531.February 17, 2020). Bone marrow was collected in syringes containing 20 U/ml sodium heparin followed by a Ficoll-Paque density gradient separation by centrifugation at 470 g for 30 min at R/T. Thereafter, mononuclear cell fraction was collected, rinsed twice with phosphate buffered saline (PBS) (Merck Life Science S.L.U. Madrid, Spain) and seeded into 75-$cm^2$ culture flasks (Merck Life Science) at $1.6 \times 10^5$ cells/$cm^2$ in Minimum Essential Medium Eagle (Thermo Fisher Scientific, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, Walkersville, MA, United States), 1% penicillin/streptomycin (P/S) (Thermo Fisher Scientific) and 1% L-glutamine (Merck Life Science). After 3 days of culture at 37°C and 5% $CO_2$, unattached cells were removed and fresh culture medium was added and replaced twice a week.

Mouse bone marrow mesenchymal stem cells (mBM-MSCs) were isolated from $\beta$-actin-GFP transgenic C57BL/6-Tg (CAG-EGFP) (The Jackson Laboratory). Briefly, mice aged 6–8 weeks were euthanized by cervical dislocation and tibias and femurs were collected and washed with PBS containing 1% P/S. Then, bone epiphyses were excised and bone marrow was flushed out using a 25-gauge needle and syringe containing low glucose

Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific). After two washing steps with PBS, BM cells were seeded into 75-cm$^2$ culture flasks at 1.6 × 10$^5$ cells/cm$^2$ and cultured in low glucose DMEM medium containing 15% FBS, 1% P/S and 1% L-glutamine following the same protocol as for human cells. When cultures were 70–80% confluent, human and mouse BM-MSCs were subcultured at 5 × 10$^3$ cells/cm$^2$ and used in passages 3–4 for subsequent experiments. Human and mouse BM-MSCs were immunophenotypically characterized by flow cytometry (FACS Canto II, BD Biosciences, San Jose, CA, United States) as previously described (Millán-Rivero et al., 2019; García-Bernal et al., 2020).

## Optic Nerve Crush

The left optic nerve was crushed at 0.5 mm from the optic disc following previously described methods (Galindo-Romero et al., 2013b; Sánchez-Migallón et al., 2018b). In brief, to access the optic nerve at the back of the eye, an incision was made in the skin overlying the superior orbital rim, the supero-external orbital contents were dissected, and the superior and external rectus muscles were sectioned. Then, the optic nerve was crushed for 10 s using watchmaker's forceps. Before and after the procedure, the eye fundus was observed through the operating microscope to assess the integrity of the retinal blood flow.

## Intravitreal Injections

All intravitreal injections were done in a final volume of 2.5 µl following previously published methods (Galindo-Romero et al., 2013b; Sánchez-Migallón et al., 2018b; Lucas-Ruiz et al., 2019c). BM-MSCs were resuspended and administered in DMEM medium at a concentration of 8 × 10$^3$ cells/µL, and other groups injected with DMEM alone were used as vehicle controls. Intravitreal administration of the $\beta$ subunit of the cholera toxin (CTB) coupled to Alexa Fluor 555 (Invitrogen, Thermofisher, Madrid Spain) was used to anterogradely trace RGC axons.

## Electroretinography

Full-field ERG was performed as described elsewhere (Alarcón-Martínez et al., 2010; Valiente-Soriano et al., 2019). Briefly, initially scotopic ERG waves were recorded binocularly from anaesthetised dark-adapted mice in response to a stimulus intensity of -4.3 (Scotopic Threshold Response), -2.5 (Rod Responseand 0.5 log cd·s/m$^2$ from a Ganzfeld dome that provided illumination of the whole retina. For the photopic study of electroretinographic waves, the animals were adapted to the light for 5 min and a background light of 30 cd/m$^2$ was used throughout the recording. Scotopic and photopic responses were recorded using Burian-Allen corneal bipolar electrodes simultaneously in both eyes. A drop of methylcellulose (Methocel 2%®; Novartis Laboratories CIBA Vision, Annonay, France) was used between the cornea and the electrodes to improve signal conductivity. The reference electrode was placed in the mouth and a needle at the base of the tail was used as a ground electrode. The electrical signals were digitized at 20 KHz using a Power Lab data acquisition board (AD Instruments, Chalgrove, United Kingdom). Standard ERG waves were analysed according to the International Society for Clinical Electrophysiology of Vision (ISCEV). For each wave, the implicit time was measured at the peak of the maximum response.

## Spectral Domain-Optical Coherence Tomography

Both retinas were analyzed under SD-OCT (Spectralis; Heidelberg Engineering, Heidelberg, Germany) adapted with a commercially available 78-D double aspheric fundus lens (Volk Optical, Inc., Mentor, OH, United States) mounted in front of the camera unit as described previously (Rovere et al., 2015; Valiente-Soriano et al., 2019). After anaesthesia, a drop of 1% tropicamide (Alcon-Cusí, S.A. Barcelona, Spain) was instilled in both eyes to induce mydriasis. Eyes were carefully kept hydrated with artificial tears and a custom-made contact permeable lens was placed on the cornea to maintain corneal hydration and clarity. Imaging was performed with a proprietary software package (Eye Explorer, version 3.2.1.0; Heidelberg Engineering). Retinas were imaged using a raster scan of 31 equally spaced horizontal B-scan. Thickness of the total, inner and outer retina was measured manually close to the optic nerve head and at 1 mm from it always in central sections spanning the optic disc. Volume of the central retina was calculated by the software after manually aligning the inner and outer retinal limits. Finally, mBM-MSC-GFP cells were visualized in vitreous with the blue light autofluorescence (BAF) mode of the OCT.

## Tissue Processing

Animals were perfused transcardially with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Retinas were prepared as flat mounts (Galindo-Romero et al., 2011). Brains were cryoprotected in increasing solutions of sucrose, embedded in Tissue-Tek (Sakura, Sakura-Finetek, Barcelona, Spain) and cryostated at 25 µm. Optic nerves were cleared using the CUBIC protocol (Susaki et al., 2014). Briefly, after washing the nerves in PBS, they were kept in scale 1 solution (Susaki et al., 2015; Liang et al., 2016) at 37°C for 4 days. Nerves were mounted in the same solution for imaging.

## Immunodetection

Immunodetection in flat mounts and brain coronal sections was carried out as reported (Galindo-Romero et al., 2011; Nadal-Nicolás et al., 2015). Primary antibodies were: mouse anti-Brn3a (1:500; MAB1585, Merck Millipore; Madrid, Spain), mouse anti-human mitochondria (1:800, ab3298 Abcam, Cambridge, United Kingdom), and rabbit anti-melanopsin (1:1,000; AB-N39 Advanced Targeting Systems ATS, Joure, Netherlands). Secondary detection was carried out with Alexa Fluor-coupled secondary antibodies (1:500; Molecular Probes; Thermo Fisher Scientific, Madrid, Spain). Retinal whole-mounts and brain coronal sections were mounted with anti-fading mounting media.

## Image Acquisition and Analyses

Images were acquired using a Leica DM6B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany). Retinal

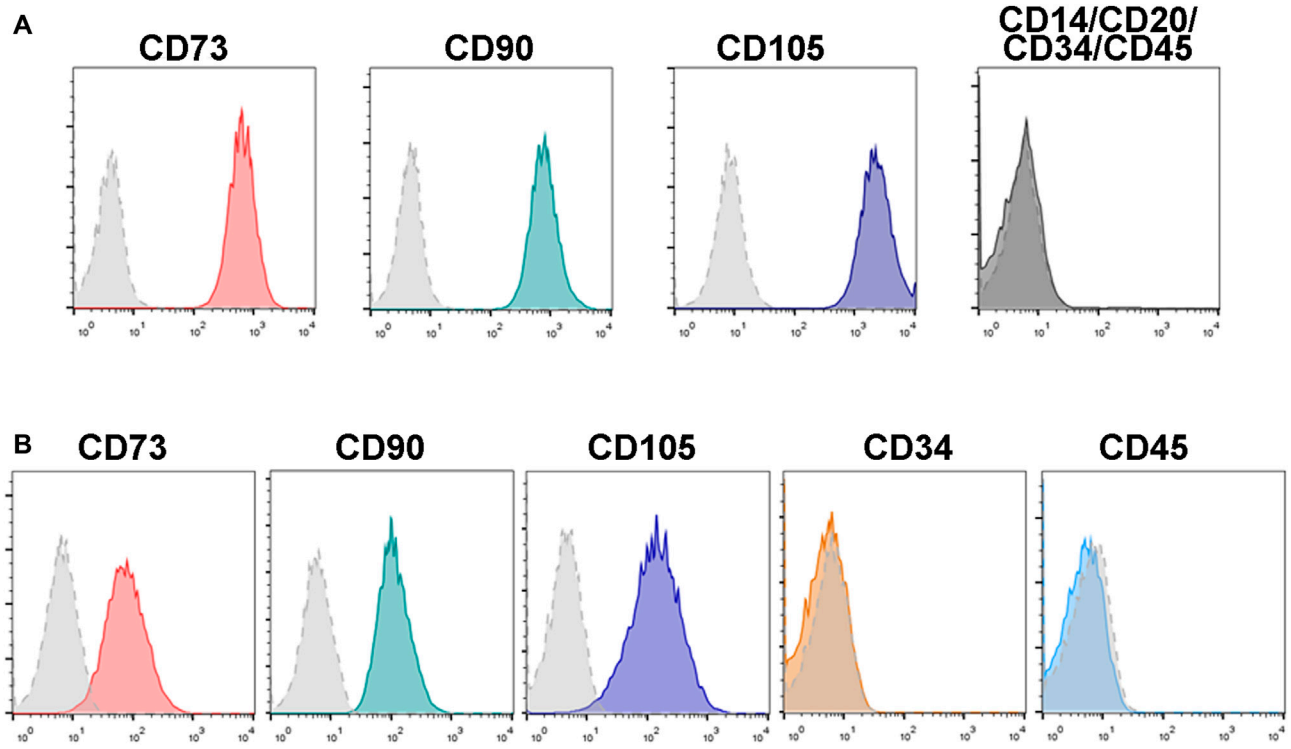


**FIGURE 2 |** Immunophenotypical analysis of human and mouse BM-MSCs. MSCs isolated from human **(A)** and mouse **(B)** bone marrow express typical MSC markers such as CD73, CD90 and CD105, whereas expression of the hematopoietic markers CD14, CD20, CD34, and CD45 are low or negative. Control isotype antibodies staining are shown as dotted light grey histograms. Histograms show representative flow cytometry results obtained from $n = 3$ separate human and mouse BM-MSC samples.

photomontages were reconstructed from individual squared images of 500 µm. Brn3a$^+$RGCs were quantified automatically and m$^+$RGCs manually dotted on the photomontages and then quantified. RGC distribution was assessed by isodensity or neighbour maps using previously reported methods (Galindo-Romero et al., 2011; Lucas-Ruiz et al., 2019b). In brief, isodensity maps show the density of RGCs with a colour scale that goes from 0–500 RGCs/mm$^2$ (purple) to ≥3,200 RGCs/mm$^2$ (red). Those maps are useful to visualize the distribution of abundant cell populations. However, to assess the topography of low number populations (i.e., m$^+$RGCs or the number of surviving RGCs long-term after axotomy) neighbour maps are better suited because they depict the number of neighbours around a given cell in a radius of 0.2 mm with a colour scale that goes from 0–2 neighbours (purple) to >21 neighbours (dark red).

## Statistical Analyses

Data were analyzed and plotted with GraphPad Prism v.7 (GraphPad, San Diego, CA, United States). Data are presented as mean ± standard deviation (SD). Differences were considered significant when $p < 0.05$. Statistical tests and number of analyzed samples are detailed in results.

# RESULTS

## Immunophenotypic Characterization of Human and Mouse Bone Marrow-Mesenchymal Stromal Cells

After isolation, both human and mouse BM-MSCs displayed a spindle-shaped fibroblastic morphology in culture. Flow cytometry immunophenotyping analyses showed that human and mouse BM-MSCs express high levels of the mesenchymal markers CD73, CD90 and CD105, and negligible expression of typical hematopoietic markers such as CD14, CD20, CD34 and CD45 (**Figure 2**).

## Human and Mouse Bone Marrow-Mesenchymal Stromal Cells Survive in the Vitreous up to 5 days After Transplantation

mBM-MSCs-GFP$^+$ were observed *in vivo* in the vitreous using the BAF mode of the SD-OCT (**Figure 3A**). Analyses in flat mounts showed that the cells did not integrate in the retina. Instead, BM-

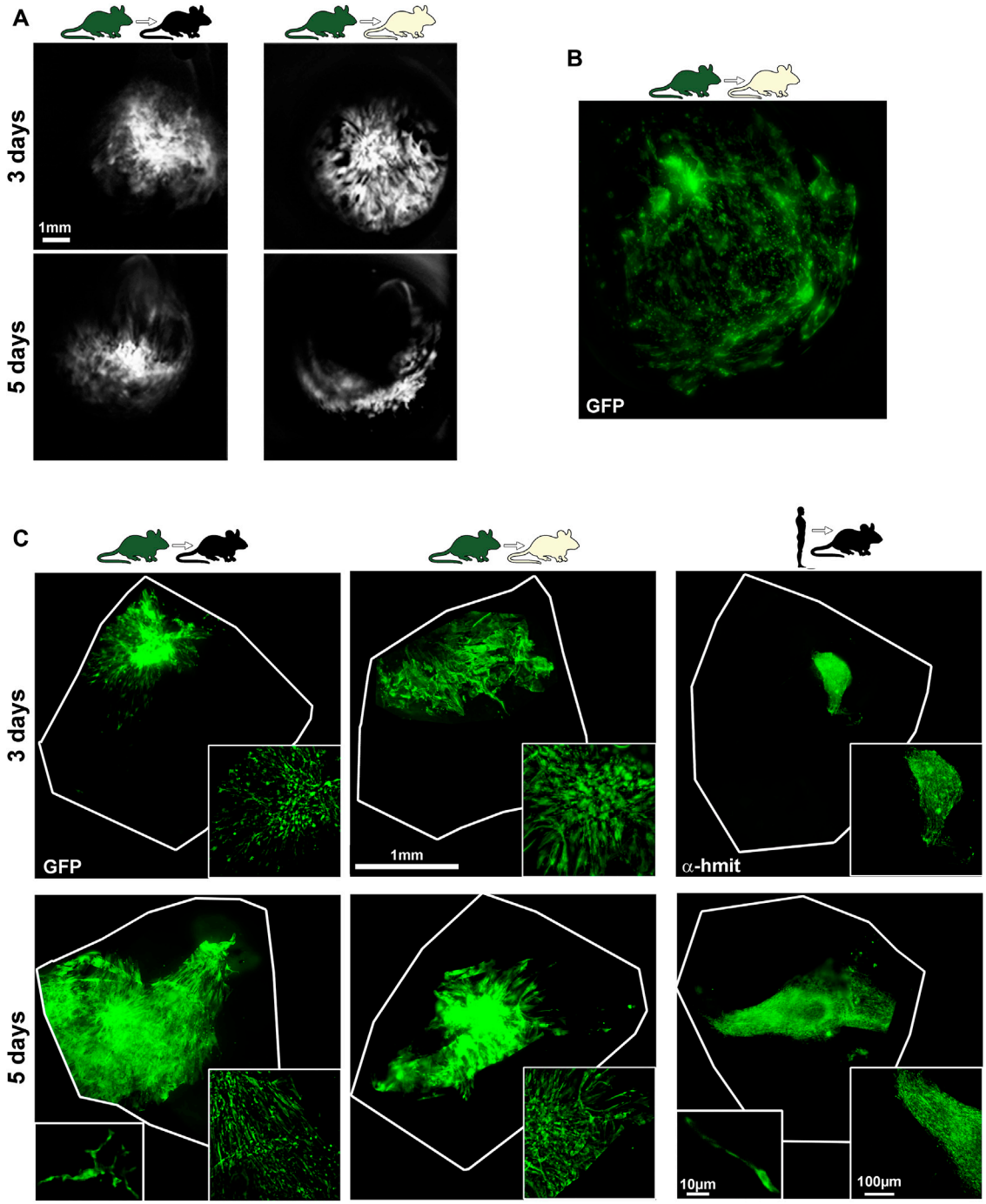


**FIGURE 3 |** Visualization of BM-MSCs. **(A)**: *In vivo* OCT images showing mBM-MSCs in the vitreous body at 3 and 5 days after syngeneic (left) and allogeneic (right) transplants. **(B,C)**: *Ex vivo*, mBM-MSCs were observed attached to the lens [**(B)**, allogeneic transplant] and on the retinal surface forming a mesh **(C)**. In **(C)**, the retinal petals where cells were injected are outlined. At the bottom right of each image is shown a magnification of the BM-MSC meshes. At the bottom left of 5 days syngraft and xenograft panels are shown high power magnifications of individual mouse and human BM-MSCs, respectively. Grafts were observed at 3 and 5, but not at 90 days. α-h-mit: anti-human mitochondrial immunostaining.

MSCs were attached to the lens (**Figure 3B**) and forming a mesh on top of the ganglion cell layer that was visible at 3 and 5 but not at 90 days (**Figure 3C**). After transplantation, human and mouse BM-MSCs displayed different morphologies: mBM-MSCs showed a branched structure, while hBM-MSCs retained the spindle-shaped morphology observed in culture. Finally, hMSCs formed tighter and more compact meshes than mBM-MSCs (**Figure 3C**, magnifications).

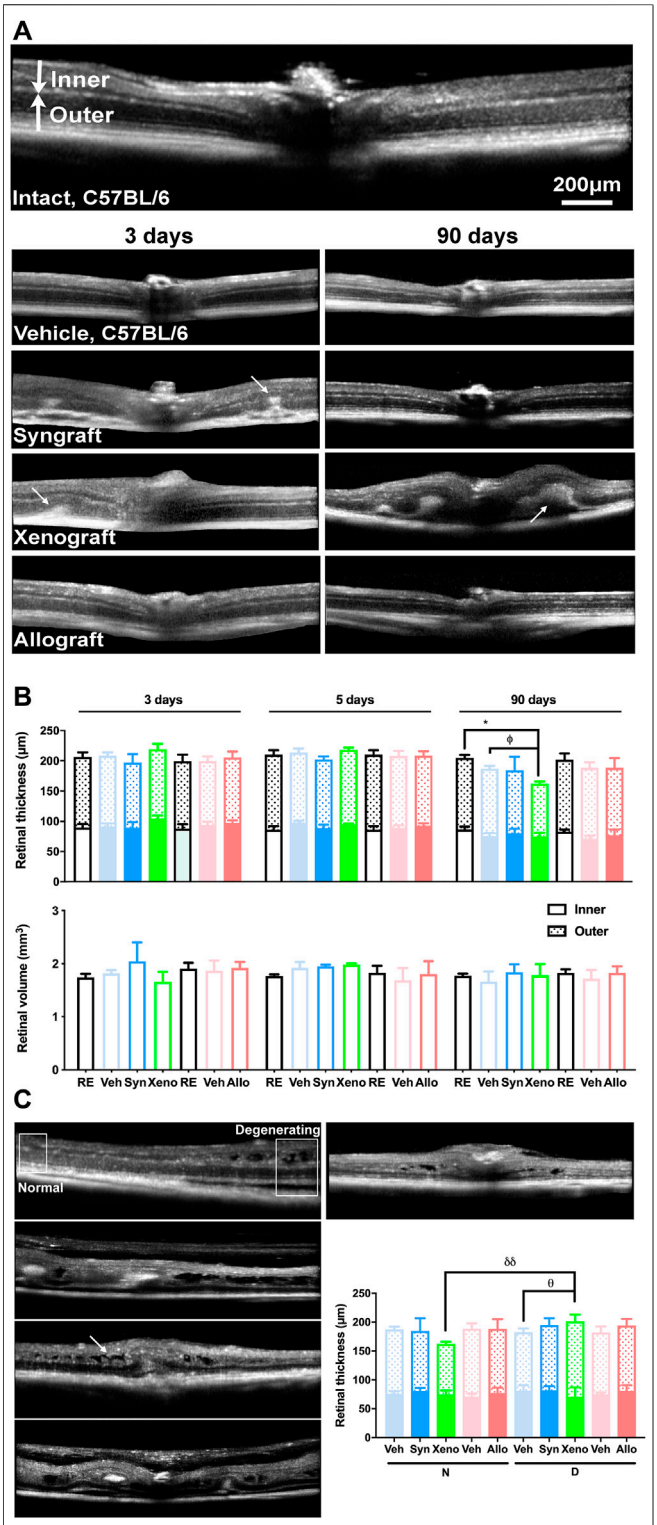


**FIGURE 4 |** Xenotransplant causes retinal edema, detachment, and thinning. **(A)**: *In vivo* OCT sections spanning the optic nerve in control (RE, 5 months old mouse), vehicle, and transplanted retinas analyzed at 3 or 90 days post-ONC. Intact and vehicle images are from C57/Bl6 mice because there was no difference between the recipient strains. In syngrafted and xenotransplanted retinas there were hyper-reflective areas below the retina (arrows) observed at 3 days that disappeared at 90 days in the syngeneic transplant but remained and grew in the xenotransplant causing edemas, subretinal fluid, retinal folding, and detachment (arrows). **(B)**: Top, stacked column graphs showing the total, inner and outer retinal thickness ± SD (µm) measured at 1 mm from the optic disc in central sections. Bottom, column graphs showing the central retinal volume ± SD ($mm^3$) in control and experimental animals. Xenografted retinas were significantly thinner than their right contralateral and vehicle-treated ones (*$p < 0.05$, compared to right eyes; $^{\theta}p < 0.05$, compared to vehicle. Kruskal-Wallis test, Dunn's multiple comparisons test) however their retinal volume did not diminish. **(C)**: *In vivo* SD-OCT sections from the central retina of 90 days xenografted animals showing different abnormalities (arrow points to retinal edemas). Framed areas in the top left image show non-degenerating (apparently normal/healthy) and degenerating regions. Because the normal areas were thinner and the degenerating areas were thicker (quantification in graph), the retinal volume did not change ($^{\theta}p < 0.05$, compared to its vehicle; $^{\delta\delta}p < 0.01$, comparing normal and degenerating areas. Non-parametric Mann-Whitney test). $n = 5$ animals/group/time point at 3 and 5 days, and $n = 4$/group at 90 days.

## Bone Marrow-Mesenchymal Stromal Cells Xenogeneic Transplant Alters the Retinal Structure

In the OCT sections of syngeneic and xenografted animals analyzed at 3 and 5 days there were hyper-reflective areas below the retina that disappeared in the syngeneic group, but progressed in the xenogeneic one, causing retinal detachment, edema and folding (**Figure 4A**, arrows). These anomalies were found mainly around or near the optic nerve head.

Retinal thickness and volume were similar between strains and did not change at 3 and 5 days in any of the experimental groups or at 90 days in the allografted and syngrafted groups. As for the xenografted retinas, they significantly thinned at 90 days, but nevertheless their volume remained within normal values (**Figure 4B**). Retinal thickness was measured at 1 mm from the optic disc, where the retinal structure was, normally, well preserved. Would volume maintenance be related to significant swelling in areas of degeneration that compensates for thinning? We measured the retinal thickness in the degenerating areas, which were usually located near the optic disc and found that indeed the xenografted retinas were significantly thicker than the rest of the groups (**Figure 4C**).

## Syngeneic Transplant of Bone Marrow-Mesenchymal Stromal Cells Neuroprotects Both Functional Subtypes of Retinal ganglion cells

In vehicle-treated retinas both functional subtypes of RGCs, vision-forming (Brn3a$^+$) and non-vision forming (M1-M3 melanopsin$^+$), underwent the course of axotomy-induced degeneration already reported (Valiente-Soriano et al., 2014; Sánchez-Migallón et al., 2018a) (**Figure 5**, **Figure 6**, and **Supplementary Figure S1**). Thus, Brn3a$^+$RGC loss was significant at 3 days and progressed steadily up to 90 days when <1% of the original population remained (**Figure 5**), while 40% of m$^+$RGCs that are more resilient to this injury (Sánchez-Migallón et al., 2018a), survived at 90 days (**Figure 6**).

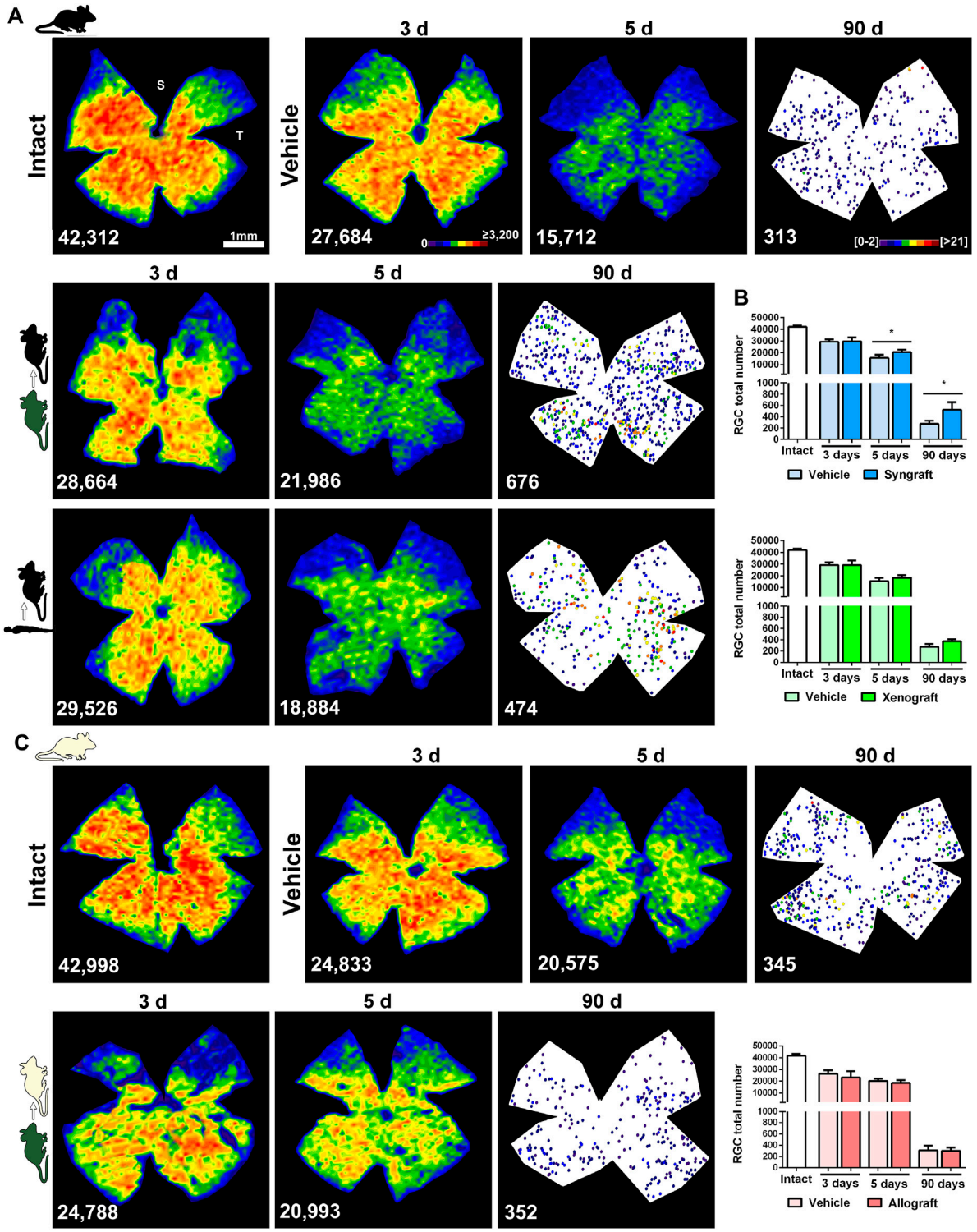


**FIGURE 5 |** Syngeneic transplant neuroprotects vision-forming RGCs. **(A)**: Representative isodensity maps (intact and 3 and 5 days after ONC) or neighbour maps (90 days after ONC) showing the distribution of RGCs in intact, ONC + vehicle, ONC + mBM-MSCs and ONC + hBM-MSC retinas from the C57/BL6 strain. **(B)**: Bar graphs showing the total number of Brn3a$^+$RGCs ± SD in syngeneic (top) and xenogeneic (bottom) transplanted retinas and their controls (*$p < 0.05$, non-parametric Mann-Whitney test). **(C)**: Representative isodensity maps (intact, and 3 and 5 days after ONC) or neighbour maps (90 days after ONC) showing the distribution of RGCs in intact, ONC + vehicle and ONC + mBM-MSCs retinas from the Balb/c strain. **(D)**: Bar graphs showing the total number of Brn3a$^+$RGCs ± SD in allogeneic transplanted retinas and their controls. At the bottom of each map is shown the number of RGCs counted in the original retina. Colour codes for isodensity and neighbour maps appear in the first row. For more details see methods. $n$ = 5 animals/group/time point at 3 and 5 days, and $n$ = 4/group at 90 days.

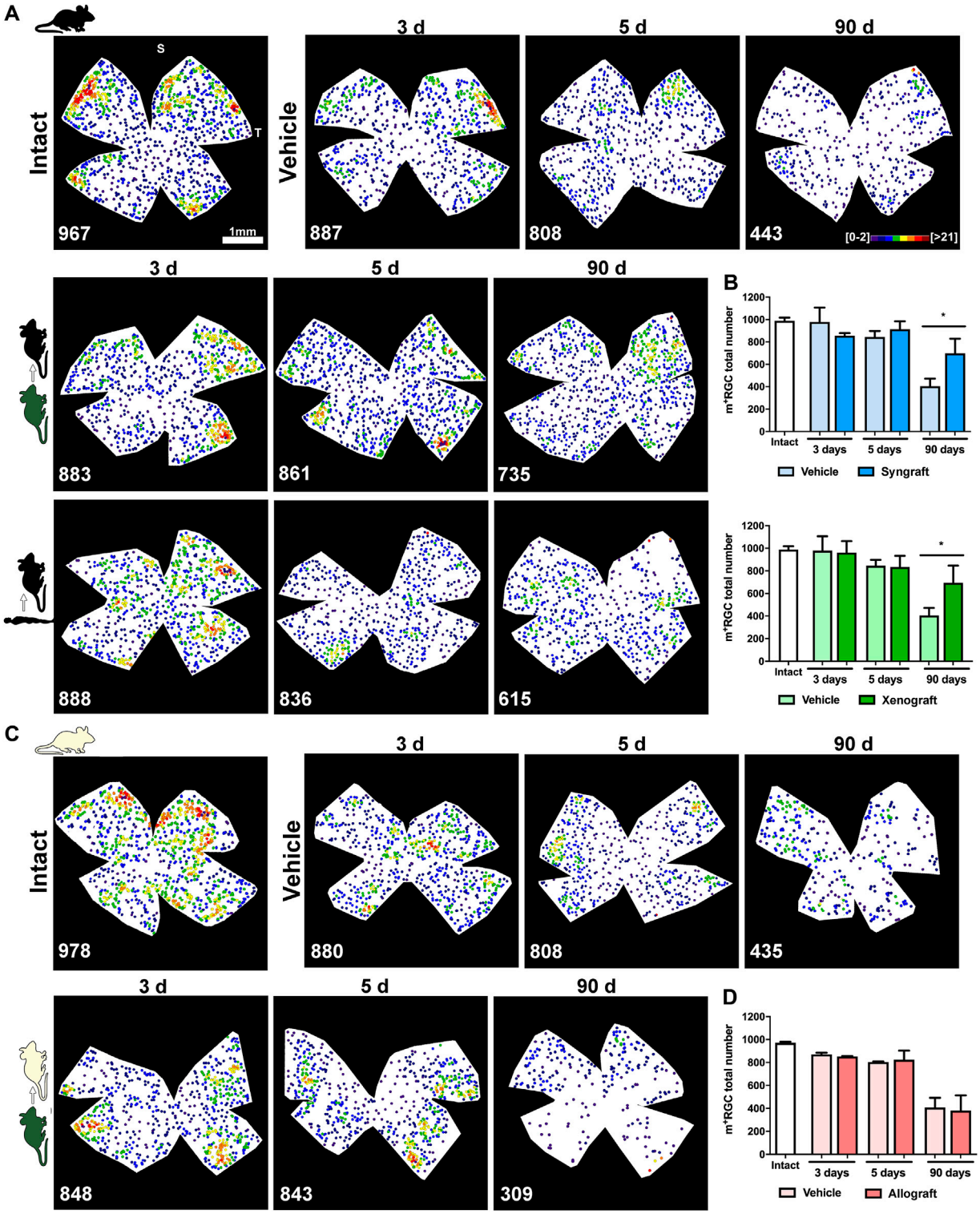


**FIGURE 6 |** Syngeneic and xenogeneic transplants neuroprotect non-vision forming RGCs. **(A)**: Representative neighbour maps showing the distribution of $m^+$RGCs in intact, ONC + vehicle, ONC + mBM-MSCs and ONC + hBM-MSC retinas from the C57/Bl6 strain. **(B)**: Bar graphs showing the total number of $m^+$RGCs ± SD in syngeneic (top) and xenogeneic (bottom) transplanted retinas and their controls (*$p < 0.05$, non-parametric Mann-Whitney test) **(C)**: Representative neighbour maps showing the distribution of $m^+$RGCs in intact, ONC + vehicle and ONC + mBM-MSCs retinas from the Balb/c strain. **(D)**: Bar graphs showing the total number of $m^+$RGCs ± SD in allogeneic transplanted retinas and their controls. At the bottom of each map is shown the number of $m^+$RGCs counted in the original retina. Colour codes neighbour maps appear in the first row. For more details see methods. $n$ = 5 animals/group/time point at 3 and 5 days, and $n$ = 4/group at 90 days.

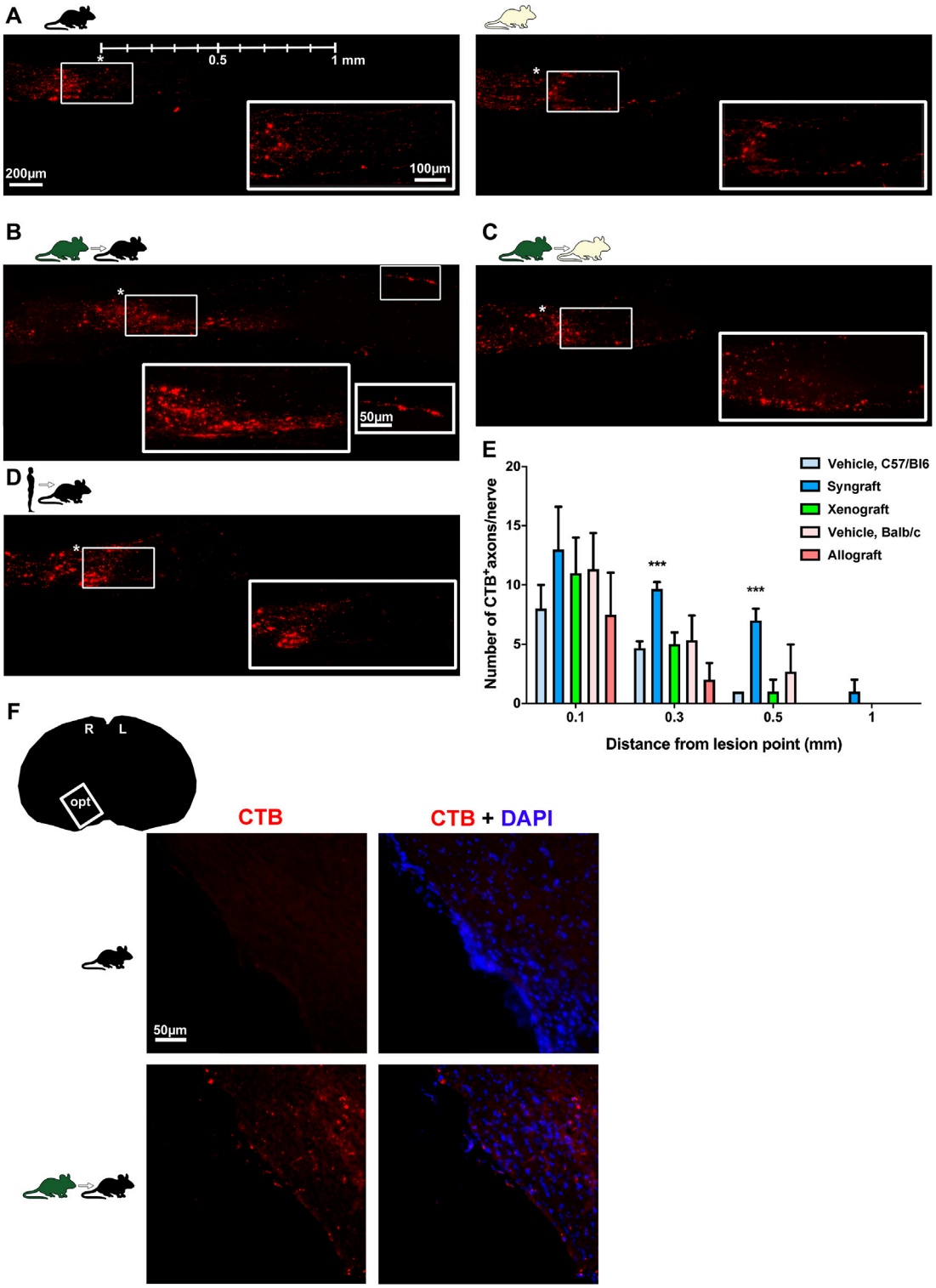


**FIGURE 7 |** Syngeneic transplant supports axonal regeneration. **(A–D)** Photomontages of cleared optic nerves showing CTB-anterogradely traced RGC axons 90 days after ONC in vehicle-treated retinas, and BM-MSC-transplanted retinas. magnifications on the bottom right are from the framed areas. Lesion site is marked with an asterisk. Rostral left, caudal, right. $n = 3$ nerves/group. **(E)**: Mean number±SD of $CTB^+$axons quantified at increasing distances from the lesion site (***$p < 0.001$ Kruskal-Wallis test, Dunn's multiple comparisons test). **(F)**: $CTB^+$axons in the optic tract were observed in one animal from the syngeneic group ($n = 3$ brains/group).

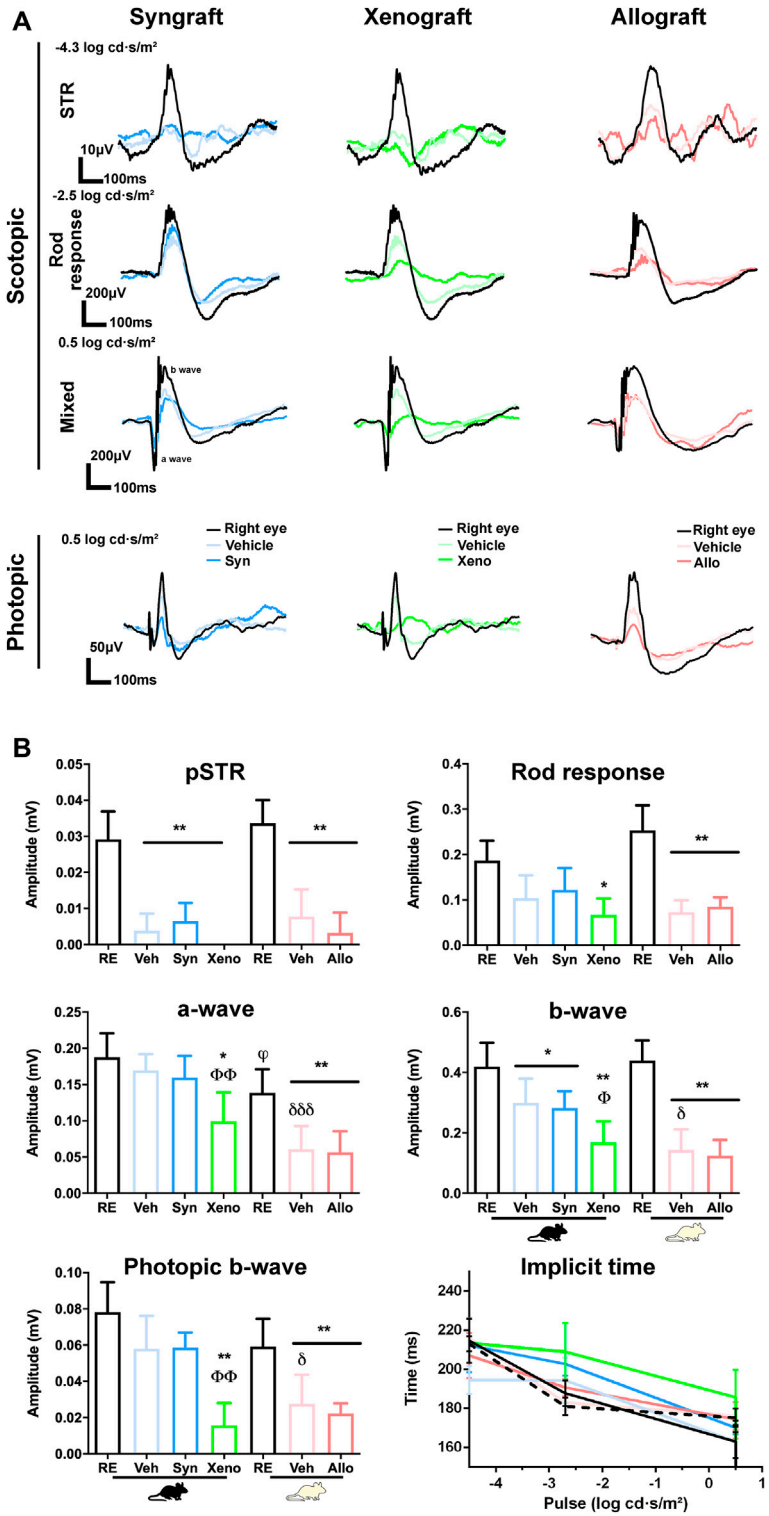


**FIGURE 8 |** Retinal functionality decreases by axotomy and is further impaired by the xenograft. Electroretinographic analysis 90 days after ONC and vehicle or BM-MSCs intravitreal administration. Right eyes were used as controls. **(A)**: ERG traces in scotopic and photopic conditions. **(B)**: Graphs showing the quantification of the positive scotopic threshold response (pSTR, RGCs), scotopic rod response (rod bipolar cells), scotopic mixed response (b-wave: cone and rod bipolar cells; a-wave: cones and rods), photopic b-wave (cone bipolar cells), and implicit time at the different light pulses. Implicit time: lines colour-coded as graphs (dashed black line: Balb/c, solid black line, C57/Bl6). RE: right eyes. Veh: ONC + vehicle. Syn: ONC + syngraft. Xeno: ONC + xenograft. Allo: ONC + allograft. Mouse silhouettes represent the recipient strain. *Experimental vs. right eyes (*$p < 0.05$, **$p < 0.01$ Kruskal-Wallis test, Dunn's multiple comparisons test). $^{\theta}$Xenografted vs. ONC + vehicle ($^{\theta\theta}p < 0.01$, non-parametric Mann-Whitney test), $^{\varphi}$albino *vs* pigmented right eyes ($^{\varphi}p < 0.05$, non-parametric Mann-Whitney test), $^{\delta}$Albino vs. pigmented ONC + vehicle eyes ($^{\delta}p < 0.05$; $^{\delta\delta\delta}$ $p < 0.001$, non-parametric Mann-Whitney test). 4 animals/group were recorded.

The syngrafts had a small but significant neuroprotective effect on Brn3a$^+$RGCs at 5 and 90 days (**Figure 5B**), a rescue that was not observed in the xenografted (**Figure 5B**) or allografted (**Figure 5C**) retinas. For m$^+$RGCs, both the syngeneic and the xenogeneic transplants were beneficial, surviving 70% of their original population at 90 days (**Figures 6A–D**).

## Regenerating Axons Distant From the Lesion Site Are Observed Only in Syngrafted Retinas

CTB-labelled axons were counted at increasing distances from the lesion site on cleared nerves (**Figures 7A–E**). Axonal regeneration was modest, but significantly higher in terms on number and distance in the syngeneic group. CTB-labelled axons were observed in the optic tract of one of the syngrafted animals (**Figure 7F**).

## Retinal Function Decreases After Axotomy and Is Further Impaired by the Xenograft

We recorded retinal function at the end of the experiment (**Figure 8A**). The positive scotopic threshold response (pSTR), that measures RGC function, significantly decreased in all experimental retinas as expected, with no differences among groups (**Figure 8B**).

Regarding the rest of the ERG waves, which are related to photoreceptors and their bipolar cells, the most drastic effect was observed in the xenografted group. While in syngrafted and allografted retinas the decrease of functionality was similar to their vehicle controls, the xenograft caused a higher loss of function than its vehicle (**Figure 8B**), reaching significance for the a-wave (cones and rods), b-wave (cone and rod bipolar cells) and photopic b-wave (cone bipolar cells). No significant changes were observed in the implicit time (b-wave) of any group, although the response in xenografted animals was delayed in some pulses.

Finally, the functional impairment observed in axotomized albino retinas (vehicle group) was always higher than in the pigmented strain, reaching significance for the mixed response and the photopic b-wave (**Figure 8B**) (Alarcón-Martínez et al., 2010).

# DISCUSSION

Treatment of neurodegenerative diseases is one of the major challenges of regenerative medicine. These are pleiotropic pathologies, from their cause to their physiological, cellular, and molecular signatures. Therefore, it seems unlikely to find a common denominator to target pharmacologically or genetically, even when narrowing by disease, because in most conditions there are a multitude of cell types affected.

Stem cells are a living medicine and produce bioactive molecules that vary according to the context in which they are grafted (Millán-Rivero et al., 2018). That is why stem cells are an exciting therapeutic avenue in neuroscience. What is needed to make stem cell therapy successful? i) cells should not induce a host response that makes them susceptible to being immunologically rejected; ii) grafted cells should remain alive long enough to rescue the compromised neurons; iii) target neurons should respond to the grafted cells, or, in other words, grafted cells must be the appropriate ones for each pathology and target cell type to obtain a beneficial therapeutic response. For cells to reach the clinic, they should be easily obtained, free of ethical concerns, and expandable *in vitro* without losing their properties. MSCs comply with these requirements.

Even then, treating patients with MSCs is difficult and clinical trials, mainly in phase III, are not being as successful as expected. There are many variables that need to be thoroughly investigated: i) are MSCs from different species the same? No. Even thought there are some similarities between human and mouse BM-MSCs (Jones and Schäfer, 2015), there are also differences in their secretomes (Harris, 1991); ii) do MSCs from the same species have the same properties? No, MSCs isolated from different tissues (Heo et al., 2016; Valencia et al., 2016; Grégoire et al., 2019), or from the same tissue but from different developmental stages (Gaetani et al., 2018), or healthy or diseased donors (Collins et al., 2014), or transplanted into different environments (Millán-Rivero et al., 2018) behave differently; iii) are preclinical studies comparable in terms of model, MSC type, and cell manufacture? are the ongoing clinical trials for CNS diseases homogeneous? again, the answer to both questions is no (Galipeau and Sensébé, 2018; Cui et al., 2019; Staff et al., 2019); iv) do the donor and host have an input in the therapeutic outcome? yes, as we have shown here, but again, to this we could add that different tissues from the same host may elicit a different response to the same MSC type.

MSCs are known for their immunomodulating properties, and secretion of paracrine factors (Khan and Newsome, 2019) both properties may be part of the therapeutic effect observed here and in other works using as model the injured retina (Zaverucha-do-Valle et al., 2011; Lucas-Ruiz et al., 2019a; Mesentier-Louro et al., 2019; Wen et al., 2021) or spinal cord (Ankeny et al., 2004; Wright et al., 2011. Reviewed in; Staff et al., 2019).

Our work agrees with previous reports showing in rats that the syngeneic transplant of BM-MSCs, enhances RGC survival and regeneration after optic nerve axotomy (Zaverucha-do-Valle et al., 2011; Mesentier-Louro et al., 2019). Our data in mouse extend further and we show that syngrafted BM-MSCs are able to rescue the two functional subtypes of RGCs, which are identified by their selective expression of Brn3a or melanopsin (Galindo-Romero et al., 2013a; Valiente-Soriano et al., 2014). Brn3a$^+$RGCs are those sending visual-forming information to the brain, and it has been known for a while that they are more vulnerable to injury that melanopsin$^+$RGCs, responsible for sending non-visual information (DeParis et al., 2012; González Fleitas et al., 2015; Nadal-Nicolás et al., 2015; Valiente-Soriano et al., 2015; Rovere et al., 2016; Vidal-Sanz et al., 2017; Sánchez-

Migallón et al., 2018a). Thus, in this model the syngeneic transplant of BM-MSCs works in two different species, mouse and rat. It is therefore, tempting to hypothesise that the autologous transplant of BM-MSCs will have a positive effect on human patients with optic neuropathies.

Xenotransplants are used in preclinical models to test therapeutic effects of human cells in animals prior translation into clinic. Works from our lab (Millán-Rivero et al., 2018) and others (Wen et al., 2021) show that even when human cells neuroprotect, they also trigger an immune response that alters the host anatomically and functionally, as we show here as well. Neuroprotection using human cells seems to be dependent on the type of MSCs and neurons: hWJ-MSCs (perinatal MSCs isolated from the umbilical cord Wharton's jelly) rescue Brn3a$^+$RGCs (Millán-Rivero et al., 2018; Wen et al., 2021) while here we show that hBM-MSCs do not rescue them but do rescue the other functional subtype, m$^+$RGCs.

In clinic, autologous transplants are preferable to allotransplants to avoid rejection and to increase the survival of the graft (Eliopoulos et al., 2005; Swanger et al., 2005). Here, we detected human and murine BM-MSCs in retinas at 3 and 5 days but not at 90 days. Therefore, BM-MSCs do not survive long-term irrespectively of the type of transplant. This may not be much of a problem because it has been proposed that the therapeutic effect of MSCs goes through a "hit and run" mechanism (Ankrum et al., 2014). Our data agree with this mechanism, because neuroprotection and axonal regeneration was observed at 90 days, even though the grafts had already disappeared.

In our model, BM-MSC syngrafts are better than allografts, the latter having no impact on the variables evaluated here. However, the scenario in the clinic is not so straightforward because it has been shown that MSCs from patients with some pathologies have an altered gene expression profile and an impaired immunomodulatory/immunosuppressive activity and stemness compared to those obtained from healthy individuals (de Oliveira et al., 2015; Alicka et al., 2019). Therefore, for patients allotransplants with cells from healthy subjects may be a better option than autografts.

Finally, there is still much research to be done to successfully translate MSC therapy to the clinic. Research should focus on isolating specific variables, as we have done here comparing the effect of the three transplantation modalities on the same injury model. There are plenty of variables to study, as abovementioned, for example the impact of the donor or the recipient. In our previous work (Millán-Rivero et al., 2018), we tested human Wharton's jelly MSCs isolated from $n = 3$ different umbilical cords, and the elicited neuroprotective effect was similar between them. In this case, the recipients were albino Sprague Dawley rats, and the human donors were different. In the clinic, patients are genetically different, except in syngeneic transplant between identical twins. Therefore, it would be very valuable to know whether the genetic background of different individuals (i.e., mice or rats of different strains) impacts the therapeutic potential of a given MSC type on a given disease model.

## CONCLUSION

This is the first study comparing the effect of the transplant type on the damaged central nervous system, using as model the axotomy of the optic nerve. Our results show that the syngeneic transplant of BM-MSCs rescues injured RGCs and promotes their regenerative capacity. Allogeneic transplantation has neither a positive nor a negative effect on the parameters measured here. The xenograft has a beneficial effect on non-vision forming RGCs but not on vision forming-RGCs, indicating that the future of MSC treatments may have to be tailored not only to the disease but also to the neuronal type. Finally, the xenotransplant induces pathological changes in the host retina, and a decrease in functionality compared to the untreated groups. Therefore, because the host response probably has an important effect on the therapeutic outcome, results of human cells in animals should be interpreted with caution.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Murcia (Murcia, Spain) and performed according to the guidelines of our Institution (approved protocols A13150201, A1320140704). Human bone marrow samples were collected after written informed consent and after the approval of the local Ethics Committee of the University Hospital Virgen de la Arrixaca (HUSA19/1531.February 17, 2020).

## AUTHOR CONTRIBUTIONS

Concept and design: MN-M, FL-R, AG-O, and MA-B. Acquisition of data: MN-M, FL-R, AG-O, and DG-B. Analysis and interpretation of data: all authors. Drafting the article: MA-B. Revising the article critically for important intellectual content: all authors. Final approval of the version to be published: all authors.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.772223/full#supplementary-material

## REFERENCES

Aggarwal, S., and Pittenger, M. F. (2005). Human Mesenchymal Stem Cells Modulate Allogeneic Immune Cell Responses. *Blood* 105, 1815–1822. doi:10.1182/blood-2004-04-1559

Ahani-Nahayati, M., Shariati, A., Mahmoodi, M., Olegovna Zekiy, A., Javidi, K., Shamlou, S., et al. (2021). Stem Cell in Neurodegenerative Disorders; an Emerging Strategy. *Int. J. Dev. Neurosci.* 81, 291–311. doi:10.1002/jdn.10101

Alarcón-Martínez, L., Avilés-Trigueros, M., Galindo-Romero, C., Valiente-Soriano, J., Agudo-Barriuso, M., Villa, P. d. l., et al. (2010). ERG Changes in Albino and Pigmented Mice after Optic Nerve Transection. *Vis. Res.* 50, 2176–2187. doi:10.1016/j.visres.2010.08.014

Alicka, M., Major, P., Wysocki, M., and Marycz, K. (2019). Adipose-Derived Mesenchymal Stem Cells Isolated from Patients with Type 2 Diabetes Show Reduced "Stemness" through an Altered Secretome Profile, Impaired Anti-oxidative Protection, and Mitochondrial Dynamics Deterioration. *J. Clin. Med.* 8, 765. doi:10.3390/jcm8060765

Ankeny, D. P., McTigue, D. M., and Jakeman, L. B. (2004). Bone Marrow Transplants Provide Tissue protection and Directional Guidance for Axons after Contusive Spinal Cord Injury in Rats. *Exp. Neurol.* 190, 17–31. doi:10.1016/j.expneurol.2004.05.045

Ankrum, J. A., Ong, J. F., and Karp, J. M. (2014). Mesenchymal Stem Cells: Immune Evasive, Not Immune Privileged. *Nat. Biotechnol.* 32, 252–260. doi:10.1038/nbt.2816

Berglund, A. K., Fortier, L. A., Antczak, D. F., and Schnabel, L. V. (2017). Immunoprivileged No More: Measuring the Immunogenicity of Allogeneic Adult Mesenchymal Stem Cells. *Stem Cel Res. Ther.* 8, 288. doi:10.1186/s13287-017-0742-8

Brown, H., and Esterházy, D. (2021). Intestinal Immune Compartmentalization: Implications of Tissue Specific Determinants in Health and Disease. *Mucosal Immunol* 14 (6), 1259–1270. doi:10.1038/s41385-021-00420-8

Chaudhary, D., Trivedi, R. N., Kathuria, A., Goswami, T. K., Khandia, R., and Munjal, A. (2018). *In Vitro* And *In Vivo* Immunomodulating Properties of Mesenchymal Stem Cells. *Recent Pat. Inflamm. Allergy Drug Discov.* 12, 59–68. doi:10.2174/1872213X12666180227105924

Coco-Martin, R. M., Pastor-Idoate, S., and Pastor, J. C. (2021). Cell Replacement Therapy for Retinal and Optic Nerve Diseases: Cell Sources, Clinical Trials and Challenges. *Pharmaceutics* 13, 865. doi:10.3390/pharmaceutics13060865

Collins, E., Gu, F., Qi, M., Molano, I., Ruiz, P., Sun, L., et al. (2014). Differential Efficacy of Human Mesenchymal Stem Cells Based on Source of Origin. *J. Immunol.* 193, 4381–4390. doi:10.4049/jimmunol.1401636

Cui, L. l., Golubczyk, D., Tolppanen, A. M., Boltze, J., and Jolkkonen, J. (2019). Cell Therapy for Ischemic Stroke: Are Differences in Preclinical and Clinical Study Design Responsible for the Translational Loss of Efficacy? *Ann. Neurol.* 86, 5–16. doi:10.1002/ana.25493

da Silva-Junior, A. J., Mesentier-Louro, L. A., Nascimento-Dos-Santos, G., Teixeira-Pinheiro, L. C., Vasques, J. F., Chimeli-Ormonde, L., et al. (2021). Human Mesenchymal Stem Cell Therapy Promotes Retinal Ganglion Cell Survival and Target Reconnection after Optic Nerve Crush in Adult Rats. *Stem Cel Res. Ther.* 12, 69. doi:10.1186/s13287-020-02130-7

de Oliveira, G. L. V., de Lima, K. W. A., Colombini, A. M., Pinheiro, D. G., Panepucci, R. A., Palma, P. V. B., et al. (2015). Bone Marrow Mesenchymal Stromal Cells Isolated from Multiple Sclerosis Patients Have Distinct Gene Expression Profile and Decreased Suppressive Function Compared with Healthy Counterparts. *Cel Transpl.* 24, 151–165. doi:10.3727/096368913X675142

DeParis, S., Caprara, C., and Grimm, C. (2012). Intrinsically Photosensitive Retinal Ganglion Cells Are Resistant to N-Methyl-D-Aspartic Acid Excitotoxicity. *Mol. Vis.* 18, 2814–2827.

Dhingra, S., Li, P., Huang, X.-P., Guo, J., Wu, J., Mihic, A., et al. (2013). Preserving Prostaglandin E2 Level Prevents Rejection of Implanted Allogeneic Mesenchymal Stem Cells and Restores Postinfarction Ventricular Function. *Circulation* 128, S69–S78. doi:10.1161/CIRCULATIONAHA.112.000324

Eliopoulos, N., Stagg, J., Lejeune, L., Pommey, S., and Galipeau, J. (2005). Allogeneic Marrow Stromal Cells Are Immune Rejected by MHC Class I- and Class II-Mismatched Recipient Mice. *Blood* 106, 4057–4065. doi:10.1182/blood-2005-03-1004

English, K., French, A., and Wood, K. J. (2010). Mesenchymal Stromal Cells: Facilitators of Successful Transplantation? *Cell Stem Cell* 7, 431–442. doi:10.1016/j.stem.2010.09.009

Escacena, N., Quesada-Hernández, E., Capilla-Gonzalez, V., Soria, B., and Hmadcha, A. (2015). Bottlenecks in the Efficient Use of Advanced Therapy Medicinal Products Based on Mesenchymal Stromal Cells. *Stem Cell Int.* 2015, 1–12. doi:10.1155/2015/895714

Figiel-Dabrowska, A., Radoszkiewicz, K., Rybkowska, P., Krzesniak, N. E., Sulejczak, D., and Sarnowska, A. (2021). Neurogenic and Neuroprotective Potential of Stem/Stromal Cells Derived from Adipose Tissue. *Cells* 10, 1475. doi:10.3390/cells10061475

Gaetani, M., Chinnici, C. M., Carreca, A. P., Di Pasquale, C., Amico, G., and Conaldi, P. G. (2018). Unbiased and Quantitative Proteomics Reveals Highly Increased Angiogenesis Induction by the Secretome of Mesenchymal Stromal Cells Isolated from Fetal rather Than Adult Skin. *J. Tissue Eng. Regen. Med.* 12, e949–e961. doi:10.1002/term.2417

Galindo-Romero, C., Avilés-Trigueros, M., Jiménez-López, M., Valiente-Soriano, F. J., Salinas-Navarro, M., Nadal-Nicolás, F., et al. (2011). Axotomy-induced Retinal Ganglion Cell Death in Adult Mice: Quantitative and Topographic Time Course Analyses. *Exp. Eye Res.* 92, 377–387. doi:10.1016/j.exer.2011.02.008

Galindo-Romero, C., Jiménez-López, M., García-Ayuso, D., Salinas-Navarro, M., Nadal-Nicolás, F. M., Agudo-Barriuso, M., et al. (2013a). Number and Spatial Distribution of Intrinsically Photosensitive Retinal Ganglion Cells in the Adult Albino Rat. *Exp. Eye Res.* 108, 84–93. doi:10.1016/j.exer.2012.12.010

Galindo-Romero, C., Valiente-Soriano, F. J., Jiménez-López, M., García-Ayuso, D., Villegas-Pérez, M. P., Vidal-Sanz, M., et al. (2013b). Effect of Brain-Derived Neurotrophic Factor on Mouse Axotomized Retinal Ganglion Cells and Phagocytic Microglia. *Invest. Ophthalmol. Vis. Sci.* 54, 974–985. doi:10.1167/iovs.12-11207

Galipeau, J., and Sensébé, L. (2018). Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell* 22, 824–833. doi:10.1016/j.stem.2018.05.004

García-Bernal, D., García-Arranz, M., García-Guillén, A. I., García-Hernández, A. M., Blanquer, M., García-Olmo, D., et al. (2020). Exofucosylation of Adipose Mesenchymal Stromal Cells Alters Their Secretome Profile. *Front. Cell Dev. Biol.* 8, 584074. doi:10.3389/fcell.2020.584074

García-Bernal, D., García-Arranz, M., Yáñez, R. M., Hervás-Salcedo, R., Cortés, A., Fernández-García, M., et al. (2021). The Current Status of Mesenchymal Stromal Cells: Controversies, Unresolved Issues and Some Promising Solutions to Improve Their Therapeutic Efficacy. *Front. Cell Dev. Biol.* 9, 650664. doi:10.3389/fcell.2021.650664

González Fleitas, M. F., Bordone, M., Rosenstein, R. E., and Dorfman, D. (2015). Effect of Retinal Ischemia on the Non-image Forming Visual System. *Chronobiol. Int.* 32, 152–163. doi:10.3109/07420528.2014.959526

Grégoire, C., Ritacco, C., Hannon, M., Seidel, L., Delens, L., Belle, L., et al. (2019). Comparison of Mesenchymal Stromal Cells from Different Origins for the Treatment of Graft-vs.-Host-Disease in a Humanized Mouse Model. *Front. Immunol.* 10, 619. doi:10.3389/fimmu.2019.00619

Gu, L.-H., Zhang, T.-T., Li, Y., Yan, H.-J., Qi, H., and Li, F.-R. (2015). Immunogenicity of Allogeneic Mesenchymal Stem Cells Transplanted via

Different Routes in Diabetic Rats. *Cell. Mol. Immunol.* 12, 444–455. doi:10.1038/cmi.2014.70

Harris, R. (1991). Medical Genetics. *BMJ* 303, 977–979. doi:10.1136/bmj.303.6808.977

Heo, J. S., Choi, Y., Kim, H.-S., and Kim, H. O. (2016). Comparison of Molecular Profiles of Human Mesenchymal Stem Cells Derived from Bone Marrow, Umbilical Cord Blood, Placenta and Adipose Tissue. *Int. J. Mol. Med.* 37, 115–125. doi:10.3892/ijmm.2015.2413

Hernández, R., Jiménez-Luna, C., Perales-Adán, J., Perazzoli, G., Melguizo, C., and Prados, J. (2020). Differentiation of Human Mesenchymal Stem Cells towards Neuronal Lineage: Clinical Trials in Nervous System Disorders. *Biomol. Ther.* 28, 34–44. doi:10.4062/biomolther.2019.065

Hoogduijn, M. J., Betjes, M. G. H., and Baan, C. C. (2014). Mesenchymal Stromal Cells for Organ Transplantation. *Curr. Opin. Organ. Transpl.* 19, 41–46. doi:10.1097/MOT.0000000000000036

Jones, E., and Schäfer, R. (2015). Where Is the Common Ground between Bone Marrow Mesenchymal Stem/stromal Cells from Different Donors and Species? *Stem Cel Res. Ther.* 6, 143. doi:10.1186/s13287-015-0144-8

Jungwirth, N., Salinas Tejedor, L., Jin, W., Gudi, V., Skripuletz, T., Stein, V. M., et al. (2018). Mesenchymal Stem Cells Form 3D Clusters Following Intraventricular Transplantation. *J. Mol. Neurosci.* 65, 60–73. doi:10.1007/s12031-018-1070-x

Kern, S., Eichler, H., Stoeve, J., Klüter, H., and Bieback, K. (2006). Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Umbilical Cord Blood, or Adipose Tissue. *Stem Cells* 24, 1294–1301. doi:10.1634/stemcells.2005-0342

Khan, R. S., and Newsome, P. N. (2019). A Comparison of Phenotypic and Functional Properties of Mesenchymal Stromal Cells and Multipotent Adult Progenitor Cells. *Front. Immunol.* 10, 1952. doi:10.3389/fimmu.2019.01952

Le Blanc, K., Tammik, C., Rosendahl, K., Zetterberg, E., and Ringdén, O. (2003). HLA Expression and Immunologic Propertiesof Differentiated and Undifferentiated Mesenchymal Stem Cells. *Exp. Hematol.* 31, 890–896. doi:10.1016/s0301-472x(03)00110-3

Li, J., Li, H., Cai, S., Bai, S., Cai, H., and Zhang, X. (2021). CD157 in Bone Marrow Mesenchymal Stem Cells Mediates Mitochondrial Production and Transfer to Improve Neuronal Apoptosis and Functional Recovery after Spinal Cord Injury. *Stem Cel Res. Ther.* 12, 289. doi:10.1186/s13287-021-02305-w

Liang, H., Akladios, B., Canales, C. P., Francis, R., Hardeman, E. H., and Beverdam, A. (2016). CUBIC Protocol Visualizes Protein Expression at Single Cell Resolution in Whole Mount Skin Preparations. *J. Vis. Exp.* (114), 54401. doi:10.3791/54401

Lucas-Ruiz, F., Galindo-Romero, C., García-Bernal, D., Norte-Muñoz, M., Rodríguez-Ramírez, K., Salinas-Navarro, M., et al. (2019a). Mesenchymal Stromal Cell Therapy for Damaged Retinal Ganglion Cells, Is Gold All that Glitters? *Neural Regen. Res.* 14, 1851–1857. doi:10.4103/1673-5374.259601

Lucas-Ruiz, F., Galindo-Romero, C., Rodríguez-Ramírez, K. T., Vidal-Sanz, M., and Agudo-Barriuso, M. (2019b). Neuronal Death in the Contralateral Uninjured Retina after Unilateral Axotomy: Role of Microglial Cells. *Int. J. Mol. Sci.* 20, 5733. doi:10.3390/ijms20225733

Lucas-Ruiz, F., Galindo-Romero, C., Salinas-Navarro, M., González-Riquelme, M. J., Vidal-Sanz, M., and Agudo Barriuso, M. (2019c). Systemic and Intravitreal Antagonism of the TNFR1 Signaling Pathway Delays Axotomy-Induced Retinal Ganglion Cell Loss. *Front. Neurosci.* 13, 1096. doi:10.3389/fnins.2019.01096

da Silva Meirelles, L., Fontes, A. M., Covas, D. T., and Caplan, A. I. (2009). Mechanisms Involved in the Therapeutic Properties of Mesenchymal Stem Cells. *Cytokine Growth Factor. Rev.* 20, 419–427. doi:10.1016/j.cytogfr.2009.10.002

Mesentier-Louro, L. A., Teixeira-Pinheiro, L. C., Gubert, F., Vasques, J. F., Silva-Junior, A. J., Chimeli-Ormonde, L., et al. (2019). Long-term Neuronal Survival, Regeneration, and Transient Target Reconnection after Optic Nerve Crush and Mesenchymal Stem Cell Transplantation. *Stem Cel Res. Ther.* 10, 121. doi:10.1186/s13287-019-1226-9

Millán-Rivero, J. E., Nadal-Nicolás, F. M., García-Bernal, D., Sobrado-Calvo, P., Blanquer, M., Moraleda, J. M., et al. (2018). Human Wharton's Jelly Mesenchymal Stem Cells Protect Axotomized Rat Retinal Ganglion Cells via Secretion of Anti-inflammatory and Neurotrophic Factors. *Sci. Rep.* 8, 16299. doi:10.1038/s41598-018-34527-z

Millán-Rivero, J. E., Martínez, C. M., Romecín, P. A., Aznar-Cervantes, S. D., Carpes-Ruiz, M., Cenis, J. L., et al. (2019). Silk Fibroin Scaffolds Seeded with Wharton's Jelly Mesenchymal Stem Cells Enhance Re-epithelialization and Reduce Formation of Scar Tissue after Cutaneous Wound Healing. *Stem Cel Res. Ther.* 10, 126. doi:10.1186/s13287-019-1229-6

Mishra, V. K., Shih, H.-H., Parveen, F., Lenzen, D., Ito, E., Chan, T.-F., et al. (2020). Identifying the Therapeutic Significance of Mesenchymal Stem Cells. *Cells* 9, 1145. doi:10.3390/cells9051145

Nadal-Nicolás, F. M., Sobrado-Calvo, P., Jiménez-López, M., Vidal-Sanz, M., and Agudo-Barriuso, M. (2015). Long-Term Effect of Optic Nerve Axotomy on the Retinal Ganglion Cell Layer. *Invest. Ophthalmol. Vis. Sci.* 56, 6095–6112. doi:10.1167/iovs.15-17195

Nadal-Nicolás, F. M., Vidal-Sanz, M., and Agudo-Barriuso, M. (2018). The Aging Rat Retina: from Function to Anatomy. *Neurobiol. Aging* 61, 146–168. doi:10.1016/j.neurobiolaging.2017.09.021

Ng, T. K., Fortino, V. R., Pelaez, D., and Cheung, H. S. (2014). Progress of Mesenchymal Stem Cell Therapy for Neural and Retinal Diseases. *World J. Stem Cell* 6, 111–119. doi:10.4252/wjsc.v6.i2.111

Oliveira, R. L., Chagastelles, P. C., Sesterheim, P., and Pranke, P. (2017). *In Vivo* Immunogenic Response to Allogeneic Mesenchymal Stem Cells and the Role of Preactivated Mesenchymal Stem Cells Cotransplanted with Allogeneic Islets. *Stem Cell Int.* 2017, 1–12. doi:10.1155/2017/9824698

Rheaume, B. A., Jereen, A., Bolisetty, M., Sajid, M. S., Yang, Y., Renna, K., et al. (2018). Single Cell Transcriptome Profiling of Retinal Ganglion Cells Identifies Cellular Subtypes. *Nat. Commun.* 9, 2759. doi:10.1038/s41467-018-05134-3

Rovere, G., Nadal-Nicolás, F. M., Agudo-Barriuso, M., Sobrado-Calvo, P., Nieto-López, L., Nucci, C., et al. (2015). Comparison of Retinal Nerve Fiber Layer Thinning and Retinal Ganglion Cell Loss after Optic Nerve Transection in Adult Albino Rats. *Invest. Ophthalmol. Vis. Sci.* 56, 4487–4498. doi:10.1167/iovs.15-17145

Rovere, G., Nadal-Nicolás, F. M., Wang, J., Bernal-Garro, J. M., García-Carrillo, N., Villegas-Pérez, M. P., et al. (2016). Melanopsin-containing or Non-melanopsin-containing Retinal Ganglion Cells Response to Acute Ocular Hypertension with or without Brain-Derived Neurotrophic Factor Neuroprotection. *Invest. Ophthalmol. Vis. Sci.* 57, 6652–6661. doi:10.1167/iovs.16-20146

Sánchez-Migallón, M. C., Valiente-Soriano, F. J., Nadal-Nicolás, F. M., Di Pierdomenico, J., Vidal-Sanz, M., and Agudo-Barriuso, M. (2018a). Survival of Melanopsin Expressing Retinal Ganglion Cells Long Term after Optic Nerve Trauma in Mice. *Exp. Eye Res.* 174, 93–97. doi:10.1016/j.exer.2018.05.029

Sánchez-Migallón, M. C., Valiente-Soriano, F. J., Salinas-Navarro, M., Nadal-Nicolás, F. M., Jiménez-López, M., Vidal-Sanz, M., et al. (2018b). Nerve Fibre Layer Degeneration and Retinal Ganglion Cell Loss Long Term after Optic Nerve Crush or Transection in Adult Mice. *Exp. Eye Res.* 170, 40–50. doi:10.1016/j.exer.2018.02.010

Serrenho, I., Rosado, M., Dinis, A., M. Cardoso, C., Grãos, M., Manadas, B., et al. (2021). Stem Cell Therapy for Neonatal Hypoxic-Ischemic Encephalopathy: A Systematic Review of Preclinical Studies. *Int. J. Mol. Sci.* 22, 3142. doi:10.3390/ijms22063142

Shabanizadeh, A., Rahmani, M. R., Yousefi-Ahmadipour, A., Asadi, F., and Arababadi, M. K. (2021). Mesenchymal Stem Cells: The Potential Therapeutic Cell Therapy to Reduce Brain Stroke Side Effects. *J. Stroke Cerebrovasc. Dis.* 30, 105668. doi:10.1016/j.jstrokecerebrovasdis.2021.105668

Song, N., Scholtemeijer, M., and Shah, K. (2020). Mesenchymal Stem Cell Immunomodulation: Mechanisms and Therapeutic Potential. *Trends Pharmacol. Sci.* 41, 653–664. doi:10.1016/j.tips.2020.06.009

Staff, N. P., Jones, D. T., and Singer, W. (2019). Mesenchymal Stromal Cell Therapies for Neurodegenerative Diseases. *Mayo Clinic Proc.* 94, 892–905. doi:10.1016/j.mayocp.2019.01.001

Susaki, E. A., Tainaka, K., Perrin, D., Kishino, F., Tawara, T., Watanabe, T. M., et al. (2014). Whole-brain Imaging with Single-Cell Resolution Using Chemical Cocktails and Computational Analysis. *Cell* 157, 726–739. doi:10.1016/j.cell.2014.03.042

Susaki, E. A., Tainaka, K., Perrin, D., Yukinaga, H., Kuno, A., and Ueda, H. R. (2015). Advanced CUBIC Protocols for Whole-Brain and Whole-Body Clearing and Imaging. *Nat. Protoc.* 10, 1709–1727. doi:10.1038/nprot.2015.085

Swanger, S. A., Neuhuber, B., Himes, B. T., Bakshi, A., and Fischer, I. (2005). Analysis of Allogeneic and Syngeneic Bone Marrow Stromal Cell Graft Survival in the Spinal Cord. *Cel Transpl.* 14, 775–786. doi:10.3727/000000005783982594

Tran, N. M., Shekhar, K., Whitney, I. E., Jacobi, A., Benhar, I., Hong, G., et al. (2019). Single-Cell Profiles of Retinal Ganglion Cells Differing in Resilience to Injury Reveal Neuroprotective Genes. *Neuron* 104, 1039–1055.e12. doi:10.1016/j.neuron.2019.11.006

Uccelli, A., Laroni, A., Laroni, A., Brundin, L., Clanet, M., Fernandez, O., et al. (2019). MEsenchymal StEm Cells for Multiple Sclerosis (MESEMS): a Randomized, Double Blind, Cross-Over Phase I/II Clinical Trial with Autologous Mesenchymal Stem Cells for the Therapy of Multiple Sclerosis. *Trials* 20, 263. doi:10.1186/s13063-019-3346-z

Urrutia, D. N., Caviedes, P., Mardones, R., Minguell, J. J., Vega-Letter, A. M., and Jofre, C. M. (2019). Comparative Study of the Neural Differentiation Capacity of Mesenchymal Stromal Cells from Different Tissue Sources: An Approach for Their Use in Neural Regeneration Therapies. *PLoS One* 14, e0213032. doi:10.1371/journal.pone.0213032

Valencia, J., Blanco, B., Yáñez, R., Vázquez, M., Herrero Sánchez, C., Fernández-García, M., et al. (2016). Comparative Analysis of the Immunomodulatory Capacities of Human Bone Marrow- and Adipose Tissue-Derived Mesenchymal Stromal Cells from the Same Donor. *Cytotherapy* 18, 1297–1311. doi:10.1016/j.jcyt.2016.07.006

Valiente-Soriano, F. J., García-Ayuso, D., Ortín-Martínez, A., Jiménez-López, M., Galindo-Romero, C., Villegas-Pérez, M. P., et al. (2014). Distribution of Melanopsin Positive Neurons in Pigmented and Albino Mice: Evidence for Melanopsin Interneurons in the Mouse Retina. *Front. Neuroanat.* 8, 131. doi:10.3389/fnana.2014.00131

Valiente-Soriano, F. J., Salinas-Navarro, M., Jiménez-López, M., Alarcón-Martínez, L., Ortín-Martínez, A., Bernal-Garro, J. M., et al. (2015). Effects of Ocular Hypertension in the Visual System of Pigmented Mice. *PLoS One* 10, e0121134. doi:10.1371/journal.pone.0121134

Valiente-Soriano, F. J., Ortín-Martínez, A., Di PierdomenicoDiGarcía-Ayuso, J. D., García-Ayuso, D., Gallego-Ortega, A., Miralles de Imperial-Ollero, J. A., et al. (2019). Topical Brimonidine or Intravitreal Bdnf, Cntf, or Bfgf Protect Cones against Phototoxicity. *Trans. Vis. Sci. Tech.* 8, 36. doi:10.1167/tvst.8.6.36

Vidal-Sanz, M., Galindo-Romero, C., Valiente-Soriano, F. J., Nadal-Nicolás, F. M., Ortin-Martinez, A., Rovere, G., et al. (2017). Shared and Differential Retinal Responses against Optic Nerve Injury and Ocular Hypertension. *Front. Neurosci.* 11, 235. doi:10.3389/fnins.2017.00235

Webb, A. E., Gerek, Z. N., Morgan, C. C., Walsh, T. A., Loscher, C. E., Edwards, S. V., et al. (2015). Adaptive Evolution as a Predictor of Species-specific Innate Immune Response. *Mol. Biol. Evol.* 32, 1717–1729. doi:10.1093/molbev/msv051

Wen, Y.-T., Ho, Y.-C., Lee, Y.-C., Ding, D.-C., Liu, P.-K., and Tsai, R.-K. (2021). The Benefits and Hazards of Intravitreal Mesenchymal Stem Cell (MSC) Based-Therapies in the Experimental Ischemic Optic Neuropathy. *Int. J. Mol. Sci.* 22, 2117. doi:10.3390/ijms22042117

Wright, K. T., Masri, W. E., Osman, A., Chowdhury, J., and Johnson, W. E. B. (2011). Concise Review: Bone Marrow for the Treatment of Spinal Cord Injury: Mechanisms and Clinical Applications. *Stem Cells* 29, 169–178. doi:10.1002/stem.570

Wu, X., Jiang, J., Gu, Z., Zhang, J., Chen, Y., and Liu, X. (2020). Mesenchymal Stromal Cell Therapies: Immunomodulatory Properties and Clinical Progress. *Stem Cel Res. Ther.* 11, 345. doi:10.1186/s13287-020-01855-9

Zaverucha-do-Valle, C., Gubert, F., Bargas-Rega, M., Coronel, J. L. L., Mesentier-Louro, L. A., Mencalha, A., et al. (2011). Bone Marrow Mononuclear Cells Increase Retinal Ganglion Cell Survival and Axon Regeneration in the Adult Rat. *Cel Transpl.* 20, 391–406. doi:10.3727/096368910X524764

Zschaler, J., Schlorke, D., and Arnhold, J. (2014). Differences in Innate Immune Response between Man and Mouse. *Crit. Rev. Immunol.* 34, 433–454. doi:10.1615/critrevimmunol.2014011600

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# Targeting Programmed Cell Death to Improve Stem Cell Therapy: Implications for Treating Diabetes and Diabetes-Related Diseases

*Qi Zhang[1], Xin-xing Wan[2], Xi-min Hu[1], Wen-juan Zhao[1], Xiao-xia Ban[1], Yan-xia Huang[1], Wei-tao Yan[1]* and Kun Xiong[1]**

[1]*Department of Anatomy and Neurobiology, School of Basic Medical Sciences, Central South University, Changsha, China,* [2]*Department of Endocrinology, Third Xiangya Hospital, Central South University, Changsha, China*

Stem cell therapies have shown promising therapeutic effects in restoring damaged tissue and promoting functional repair in a wide range of human diseases. Generations of insulin-producing cells and pancreatic progenitors from stem cells are potential therapeutic methods for treating diabetes and diabetes-related diseases. However, accumulated evidence has demonstrated that multiple types of programmed cell death (PCD) existed in stem cells post-transplantation and compromise their therapeutic efficiency, including apoptosis, autophagy, necroptosis, pyroptosis, and ferroptosis. Understanding the molecular mechanisms in PCD during stem cell transplantation and targeting cell death signaling pathways are vital to successful stem cell therapies. In this review, we highlight the research advances in PCD mechanisms that guide the development of multiple strategies to prevent the loss of stem cells and discuss promising implications for improving stem cell therapy in diabetes and diabetes-related diseases.

**Keywords: programmed cell death, stem cell, apoptosis, pyroptosis, necroptosis, diabetes**

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****Correspondence:***
*Kun Xiong*
*xiongkun2001@163.com*
*Wei-tao Yan*
*beakeyywt@126.com*

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## INTRODUCTION

Stem cells (SCs) are unique cell populations distinguished by the capacity of self-renewal and differentiation (Biswas and Hutchins, 2007; McElhinney et al., 2020). These unique features of SCs make them the preferred candidate for tissue repairing (Yang et al., 2019; Yang et al., 2020; Qin et al., 2021). According to different developmental stages, SCs can be categorized into distinct types, such as embryonic SCs (ESCs), induced pluripotent SCs (IPSCs), and adult SCs (ASCs) (Bogliotti et al., 2018; Hu et al., 2021). These SCs are widely utilized for regenerative medicine therapies (Gurusamy et al., 2018; Fatima et al., 2019).

The worldwide shortage of pancreas donors remains a major hurdle to islet transplantation, and SC therapy represents a highly promising alternative approach for treatments of advanced diabetes (Saleem et al., 2019; Chen et al., 2020). In SC therapy for type 1 diabetes mellitus (T1DM), insulin-producing cells can be generated from SCs (Manzar et al., 2017; Chen et al., 2020). Neural SCs (NSCs), bone marrow-derived mesenchymal SCs (BM-MSCs), and umbilical cord MSCs (UC-MSCs) are a promising treatment for diabetic retinopathy and foot ulcers (Zhang et al., 2017; Zhao et al., 2020a; Huang Q. et al., 2021).

However, the cell death of SC post-transplantation creates significant challenges to transplantation therapy (Mastri et al., 2014). According to different death processes, cell

death are categorized as: programmed cell death (PCD), a precise and genetically controlled cellular death, and non-PCD, also called necrosis (Cheng et al., 2018; Guo LM. et al., 2020; Bedoui et al., 2020). Extensive pharmacological and genetic strategies have been developed to inhibit PCD to prevent cell loss and thus improve physiological function of organs (Wang Z. et al., 2018; Yuan et al., 2019; Wu X. et al., 2020; Yan W.-T. et al., 2021). Increasing evidence indicates a close link between PCDs and cell death of transplanted SCs (Ho et al., 2017; Wang R. et al., 2020; Pierozan et al., 2020). More importantly, targeting these PCDs shows promising therapeutic effects for diabetes and diabetes-related diseases (Zhang K. et al., 2019; Hu et al., 2019).

## Distinct Forms of PCD IN SC for Transplantation

### Apoptosis

Apoptosis is characterized by the breaking up of cell in apoptotic bodies (Nikoletopoulou et al., 2013). In intrinsic pathway of apoptosis, DNA damage can activate p53, and subsequently induce genes involved in apoptosis signaling and execution (**Figure 1A**) (Hafner et al., 2019). In human ESCs, the stabilization of p53 can suppress the pluripotency of SCs after DNA damage responses (Zhang et al., 2014). In addition, silencing of the proapoptotic gene *Puma*, which is responsible for p53-dependent apoptosis, can increase pluripotency of iPSCs (Lake et al., 2012; Fu et al., 2020). Moreover, proapoptotic BCL-2 signals and ASPP1, an apoptosis-stimulating protein of p53, contributed to the induction of apoptosis in HSCs (Yamashita et al., 2015; 2016).

The extrinsic pathway of apoptosis is initiated by docking of death ligands of tumor necrosis factor (TNF) to TNF receptors 1 (TNFR1) (Carneiro and El-Deiry, 2020). TNF-α can induce apoptosis in NSCs by upregulating the phosphatidylinositol p38 mitogen-activated protein kinase (p38 MAPK) pathway (Chen et al., 2016). In HSC transplantation for treating malignancies, activation of TNF-α-TNFR1 signaling pathway caused accumulation of reactive oxygen species (ROS) in HSCs and subsequent cell damage (Ishida et al., 2017). In contrast, TNF-α-TNFR2 signaling is important for survival and function of MSCs and endothelial stem/progenitor cells (EPCs), and its deficiency resulted in reduced proliferation rate and diminished immunomodulatory effect of these cells (Beldi et al., 2020a; Beldi et al., 2020b; Naserian et al., 2020; Nouri Barkestani et al., 2021; Razazian et al., 2021).

## Autophagy

Autophagy is a self-degradative process that contributes to removing excessive or misfolded proteins and clearing damaged organelles at critical times (**Figure 1B**) (Glick et al., 2010; Andrade-Tomaz et al., 2020). The autophagy is triggered by upregulation of AMP activated protein kinase (AMPK) and downregulation of mammalian target of rapamycin complex 1 (mTORC1) (Kim et al., 2011). In ESCs and HSCs, the regulation of AMPK and mTOR kinase is essential to their homeostasis, self-renewal and pluripotency (Huang et al., 2009; Gong et al., 2018; Suvorova et al., 2019). Additionally, the precise regulation of mTOR by Sox2 is vital to reprogramming of somatic cells to form iPSCs (Wang S. et al., 2013). The viability and stemness of NSCs and ESCs were also associated with LC3 lipidation, autophagic flux, and formation of autophagosomes (Bialik and Kimchi, 2010; Vázquez et al., 2012; Gu et al., 2019; Wang et al., 2019). Additionally, the autophagy-related gene *ATG3* was shown to be a pivotal regulator of mitochondrial homeostasis regulation in ESCs (Liu et al., 2016).

Notably, Dou *et al.* demonstrated that an amyloid binding peptide with three chaperone-mediated autophagy motifs significantly reduced Aβ oligomers in iPSC cortical neurons (Dou et al., 2020). Autophagy driven by FOXO3A and FOXO1 also protected HSCs from metabolic stress and guarded ESC identity (Warr et al., 2013; Liu et al., 2017). More importantly, the coordination of autophagy and apoptosis is vital to maintaining homeostasis in BM-MSCs (Zhang et al., 2016).

## Necroptosis

Necroptosis is a programmed form of necrosis mediated by receptor interacting protein kinase 1/3 (RIPK1/3) and mixed lineage kinase domain-like (MLKL) proteins (**Figure 1C**) (Wang M. et al., 2020; Font-Belmonte, 2020; Yan WT. et al., 2021; Liao et al., 2021). Necroptosis of intestinal SCs triggered bowel inflammation in the pathogenesis of inflammatory bowel disease (Wang R. et al., 2020). In addition, compression triggered necroptosis of nucleus pulposus-derived SCs and inhibiting necroptosis rescued regeneration of degenerated intervertebral discs (Hu B. et al., 2020). Furthermore, inhibition of necroptosis is a novel strategy for allogeneic HSCs transplantation and spermatogonial SC-based therapy for male fertility preservation (Matsuzawa-Ishimoto et al., 2017; Xie et al., 2020). Moreover, cellular transplant therapy based on human olfactory SCs ameliorated motor function in Huntington's disease by preventing necroptosis (Bayat et al., 2021). Intriguingly, TNF-α also could function as a pro-regeneration factor in HSCs that primarily prevented necroptosis rather than apoptosis by activating a p65-nuclear factor κB-dependent gene program (Yamashita and Passegué, 2019).

## Pyroptosis

Pyroptosis is specific PCD mediated by cleavage of gasdermin D (GSDMD) to form membrane pores and activation of cytokines (**Figure 1D**) (Chu et al., 2020; Chen Y. et al., 2021; Huang Y. et al., 2021). Pyroptosis contributed to the cell death of human cardiac SCs (hCSCs) in an acute hyperglycemic microenvironment, which impaired cardiac regeneration in diabetic hearts (Yadav et al., 2020). The culture media collected from pyroptotic bone marrow-derived macrophages also induced pyroptosis in MSCs (Zhang C. et al., 2020). Additionally, the pyroptosis of neural progenitor cells represented a therapeutic target in Zika virus-induced brain atrophy (He Z. et al., 2020). Moreover, chitosan thermosensitive hydrogel enhanced the therapeutic efficacy of

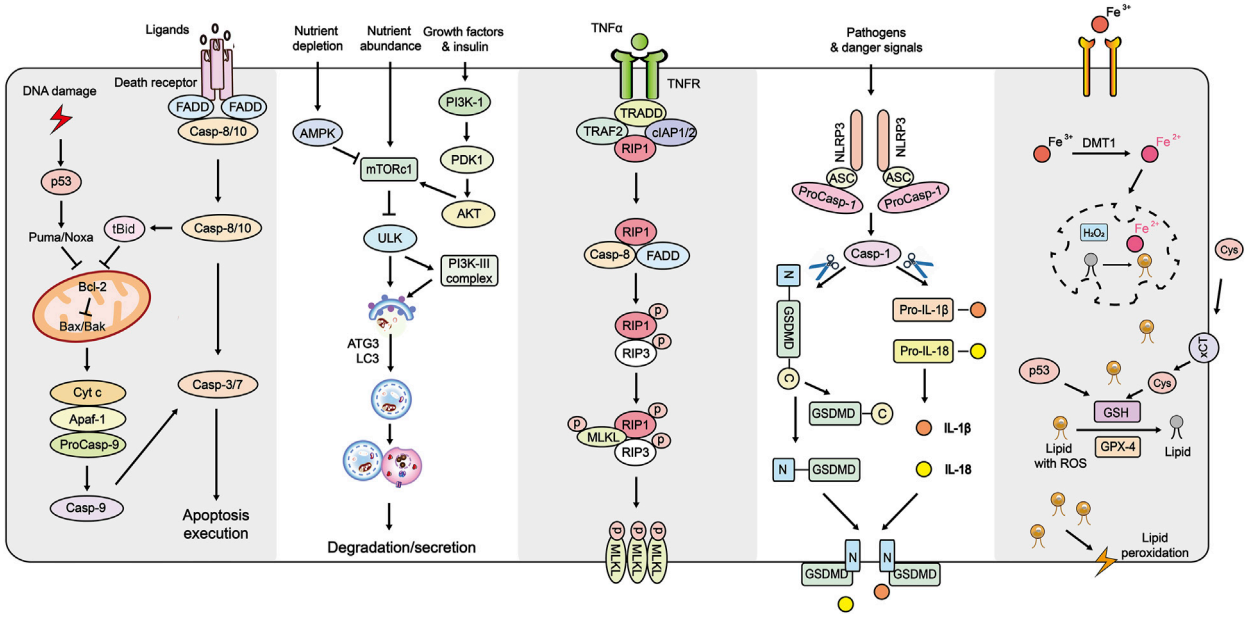


**FIGURE 1 |** A brief overview of the main molecular mechanisms in PCDs **(A)** Apoptosis is initiated by the intrinsic and extrinsic pathways. In the intrinsic pathway, DNA damage activates p53, and subsequently activates Puma/Noxa to induces signaling genes including proapoptotic proteins (Bax, Bak, tBid), apoptosome (Cyt c, Apaf-1, pro-Caspase-9), antiapoptotic protein (Bcl-2), and apoptosis execution factors (caspase-3/7). The extrinsic pathway of apoptosis is initiated by the binding of TNF to its receptors, leading to the recruitment of FADD and caspase-8. In addition, caspase-8 can cleave Bid to t-Bid, which participates in the intrinsic pathway of apoptosis **(B)** Autophagy is initiated by nutrient sensoring, including AMPK, PI3K-1, mTORC1. Autophagosome formation and maturation are mediated PI3K-III complex, ATG3, and LC3. Finally, the autophagosome contents undergo is degraded by lysosomes **(C)** Necroptosis is initiated by the binding of TNF-α and the receptor TNFR. Under conditions of caspase-8 (initiator caspase of extrinsic apoptosis) is not active, the formation of necroptosome (integration of phosphorylated RIPK1 and RIPK3) induces the MLKL phosphorylation and oligomerization. Finally, the MLKL oligomers translocation to membranes and disrupt it to kill cells **(D)** Pyroptosis is triggered by various pathogens and danger signals. These signals activate NLRP3 inflammasome, which consists of NLRP3, ASC and procaspase-1, and subsequently leads to cleavage of GSDMD and pro-IL-1β and pro-IL-18. Finally, the N-terminal fragment of GSDMD targets to membrane to form membrane pores and induces inflammatory cell death **(E)** Ferroptosis is triggered by severe lipid peroxidation with ROS and iron overload, leading to membrane damage. The uptake of $Fe^{2+}$ is regulated by DMT1. The lipid peroxidation is mainly caused by loss of activity of GSH and GPX4. The xCT also functions in regulating ferroptosis *via* Cys. In addition, p53, an initiator of intrinsic apoptosis, controls ferroptosis by regulation of the production of GSH. Abbreviations: Cyt c, cytochrome c; Apaf-1, apoptotic peptidase activating factor 1; Bcl-2, B cell chronic lymphocytic leukaemia/lymphoma-2; FADD, Fas-associated death domain; AMPK, AMP activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; PI3K-1, phosphatidylinositol 3 kinase-1; ATG3, autophagy-related gene 3; LC3, light chain 3; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF2, TNF receptor associated factor 2; TRADD, TNFR1-associated death domain protein; cIAP1/2, cell inhibitor of apoptosis protein-1/2; RIPK1/3, receptor interacting protein kinase 1/3; MLKL, mixed lineage kinase domain-like protein; NLRP3, nod-like receptor protein-3; ASC, apoptosis-associated speck-like protein; GSDMD, gasdermin D; IL, interleukin; ROS, reactive oxygen species; DMT1, divalent metal ion transporter 1; xCT, cystine/glutamate antiporter SLC7A11; Cys, cystine; GSH, glutathione; GPX4, glutathione peroxidase 4.

BM-MSCs for myocardial infarction by alleviating pyroptosis of vascular endothelial cells (Liu Y. et al., 2020). Finally, emerging evidence showed that SCs and SC-derived exosomes inhibited pyroptosis and could be used to treat different diseases (Zhang J. et al., 2020; Yan et al., 2020; Chen M.-T. et al., 2021).

## Ferroptosis

Ferroptosis is an iron-dependent form of cell death (**Figure 1E**) (Stockwell et al., 2017). The iPSC-derived cell modeling of neuroferritinopathy revealed that iron-dependent ferroptosis has a primary role in neuronal aging and degeneration (Cozzi et al., 2019). Also, iron overload (IOL) may induce cellular toxicity in hematopoietic SCs therapy for hematologic malignancies, and IOL reduction may improve outcomes (Leitch et al., 2017). In addition, catecholic flavonol quercetin inhibited erastin-induced ferroptosis in BM-MSCs (Li et al., 2020). Moreover, in Pelizaeus-Merzbacher disease, the mutant oligodendrocytes of patients exhibited the hallmarks of ferroptosis, and gene correction in patient-derived iPSCs rescued the iron-induced cell death (Nobuta et al., 2019).

The characteristics of distinct kinds of PCD in transplanted SCs provide an in-depth understanding of cell death in SC therapy. Based on the key mediators and crosstalk identified in PCD, the development of highly precise strategies to improve SC survival is possible.

## Which Types of PCD Reported IN SC can be Used to Treat Diabetes and Diabetes-Related Diseases?

Whereas SC therapy represents a highly promising therapeutic strategy for treating diabetes, PCD existed in

**TABLE 1 |** Current strategies to prevent PCD of SC for transplantation.

| Strategy | Method | Targeting PCD | SC | Application | References |
|---|---|---|---|---|---|
| **Preconditioning** | | | | | |
| Hypoxia | 1% $O_2$ for 48 h | Apoptosis | AD-MSCs | Tissue regeneration | Liu et al. (2013) |
| | 1.5% $O_2$ for 24 h | Apoptosis | MSCs | Idiopathic pulmonary fibrosis | Lan et al. (2015) |
| | 5% $O_2$ for 6 h | Apoptosis; autophagy | BM-MSCs | Diabetic lower-limb ischemia | Liu et al. (2015a) |
| | 5% $O_2$ for 48 h | Apoptosis | BM-MSCs | Diabetic lower-limb ischemia | Liu et al. (2015b) |
| Oxidative stress | 100 mM $H_2O_2$ for 2 days | Apoptosis | Cardiac progenitor cells | Heart failure | Pendergrass et al. (2013) |
| | 50 μM $H_2O_2$ for 12 h | Apoptosis | BM-MSCs | Wound healing | Guo et al. (2020b) |
| Heat shock | 42 C for 1 h | Apoptosis | UC-MSCs | Acute lung injury | Lv et al. (2021) |
| | 42 C for 1 h | Apoptosis | BM-MSCs | Premature ovarian failure | Chen et al. (2018) |
| Lipopolysaccharide | 1.0 l g/mL for 24 h | Apoptosis | BM-MSCs | Hypoxia and serum deprivation | Wang et al. (2013a) |
| Melatonin | 5 μM for 24 h | Apoptosis | BM-MSCs | Ischemic kidney | Mias et al. (2008) |
| Oxytocin | 10 nM for 24 h | Apoptosis | BM-MSCs | Hypoxia and serum deprivation | Noiseux et al. (2012) |
| Sevoflurane | 2 vol% for 2 h | Apoptosis | BM-MSCs | Hypoxia and serum deprivation | Sun et al. (2014) |
| Resveratrol | 10 μM for 10 h | Autophagy | ESCs | Enhancing pluripotency of SC | Suvorova et al. (2019) |
| | 10 μM for 2 h | Apoptosis | ADSC | Type 1 diabetes | Chen et al. (2019b) |
| **Genetic modification** | | | | | |
| AURKA | Lentivirus vectors transfection | Apoptosis; autophagy | ADSC | Diabetic wound healing | Yin et al. (2020) |
| $VEGF_{165}$ | Bi-Tet transfection | Apoptosis | ESCs | Cardiac function | Xie et al. (2007) |
| HGF | Adenoviral vector transfection | Apoptosis | UC-MSCs | Acute liver failure | Tang et al. (2016) |
| | Adenoviral vector transfection | Apoptosis | BM-MSCs | Hepatocirrhosis | Zhang et al. (2018) |
| ERBB4 | Lentivirus vectors transfection | Apoptosis | MSCs | Myocardial infarction | Liang et al. (2015) |
| HIF1α | Lentivirus vectors transfection | Apoptosis | ADSC | Diabetic wound healing | Xu et al. (2020) |
| | Adenoviral vector transfection | Apoptosis | MSCs | Myocardial infarction | Huang et al. (2014) |
| **3D cell culturing** | | | | | |
| 3D-dynamic system | Culturing for 48 h | Apoptosis | BM-MSCs | Myocardial infarction | Wang et al. (2018a) |
| 3D floating culture | Culturing for 3 days | Apoptosis | MSCs | Enhancing survival of SC | Komatsu et al. (2020) |
| 3D organ culture | Culture with D-serine and RA for 3 weeks | Apoptosis | Spermatogonial SCs | Spermatogenesis | Modirshanechi et al. (2020) |
| **Co-transplantation** | | | | | |
| NSCs and OECs | NSCs: OECs = 1:1 | Apoptosis | NSCs | Traumatic brain injury | Liu et al. (2014) |
| EPI-NCSCs and OECs | EPI-NCSCs: OECs = 1:1 | Apoptosis | EPI-NCSCs | Peripheral nerve injury | Zhang et al. (2019b) |
| BM-MSCs and monocytes | BM-MSCs: monocytes = 1:30 | Apoptosis | BM-MSCs | Facial nerve axotomy | Wu et al. (2020a) |

*Abbreviations: SCs, stem cells; MSCs, mesenchymal stem cells; ESCs, embryonic stem cells; AD-MSCs, adipose-derived mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; UC-MSCs, umbilical cord-derived mesenchymal stem cells; ADSC, Adipose-derived stem cells; NSCs, neural stem cells; EPI-NCSCs, epidermal neural crest stem cells; OECs, olfactory ensheathing cells; RA, retinoic acid; AURKA, Aurora kinase A; $VEGF_{165}$, vascular endothelial growth factor 165; HGF, hepatocyte growth factor; ERBB4, v-erb-b2, avian erythroblastic leukemia viral oncogene homolog 4; HIF1α, hypoxia-inducible factor 1α.*

SCs hinders the therapeutic effects (Saleem et al., 2019). For example, hyperglycemia increased apoptosis of Adipose-derived SCs (ADSCs) and decreased their paracrine function in diabetic retinopathy (Hajmousa et al., 2016; Xu et al., 2020). Furthermore, BM-MSCs from streptozotocin-induced diabetic rats showed impaired antiapoptosis, proliferation and paracrine abilities (Jin et al., 2010).

More importantly, overexpression of hypoxia-inducible factor 1α (HIF1α), a regulator of oxygen homeostasis, significantly alleviated the ADSC apoptosis rate and enhanced diabetic wound closure (Xu et al., 2020). Norepinephrine can also reverse high glucose-induced apoptosis in MSCs through the AKT/BCL-2 pathway (Kong et al., 2019). The peroxisome proliferator-activated receptor- $\gamma$ agonist pioglitazone (PGZ) is used for management of diabetes (Cho et al., 2019). It was reported that PGZ had a protective effect on compression-mediated apoptosis in MSCs by suppressing mitochondrial apoptosis pathway (Hu et al., 2019).

In addition, autophagy played a protective role in ADSC under high glucose stress (Li et al., 2018). More importantly, the overexpression of Aurora kinase A (AURKA), a cell cycle-regulated kinase, enhanced autophagy of ADSCs, decreased apoptosis, and promoted wound healing in diabetic mice (Yin et al., 2020). Also, the inhibition of autophagy significantly promoted high glucose/ROS-mediated apoptosis in ADSCs (Li

et al., 2018). Furthermore, upregulating autophagy in periodontal ligament SCs partially recovered periodontium tissues in a diabetic rat periodontal trauma model, suggesting the protective role of autophagy for SC transplantation (Zhang K. et al., 2019). Additionally, exosomes derived from MSCs ameliorated type 2 diabetes by activating autophagy *via* AMPK pathway (He Q. et al., 2020). Moreover, pyroptosis contributed to the cell death of hCSCs in an acute hyperglycemic microenvironment, which impaired cardiac regeneration in diabetic hearts (Yadav et al., 2020).

Although emerging evidence indicates that many forms of PCD play vital roles in the cell death of SCs for treating diabetes and diabetes-related diseases, the identification of necroptosis and ferroptosis remains to be explored further.

## Current Strategies to Prevent PCD OF SC for Transplantation

### Preconditioning

The benefit of preconditioning of SC was first described in ischemic myocardium, and to date, a variety of preconditioning strategies have been shown to improve SC survival (**Table 1**) (Sart et al., 2014; Hu and Li, 2018). Hypoxic preconditioning could decrease apoptosis and increased autophagy in MSCs and BM-MSCs (Liu et al., 2013; Liu et al., 2015a; Lan et al., 2015). Exposure to oxidative stress decreased apoptosis of BM-MSCs upon serum withdrawal and oxidative stress (Pendergrass et al., 2013; Guo L. et al., 2020). Furthermore, heat shock pretreatment enhanced repair effects of MSCs for acute lung injury and premature ovarian failure *via* reducing apoptosis and macrophages (Chen et al., 2018; Lv et al., 2021).

Preconditioning of SCs with pharmacological or chemical agents also improved SCs survival *via* preventing PCD. For example, lipopolysaccharide (LPS) preconditioning protected MSCs against apoptosis induced by hypoxia and serum deprivation *via* suppressing the extracellular signal-regulated kinase signaling pathway (Wang J. et al., 2013; Hu and Li, 2018). Preconditioning with melatonin, oxytocin, and sevoflurane also increased the resistance of MSCs to apoptosis and their paracrine activity (Mias et al., 2008; Noiseux et al., 2012; Sun et al., 2014). In addition, pretreatment with resveratrol induced autophagy in ESCs *via* activation of AMPK/ULK1 pathway (Suvorova et al., 2019). Moreover, TNF-α and other inflammatory mediators preconditioning could increase the survival, proliferation and immunomodulatory effects of MSCs and EPCs (Song et al., 2019; Beldi et al., 2020a; Ferreira et al., 2021; Nouri Barkestani et al., 2021).

## Genetic Modification

Accumulated studies have identified promising therapeutic molecular targets for genetic modification to prevent PCD of SCs. Regarding cardiovascular disease, ESCs transfected with inducible VEGF inhibited apoptosis of transplanted cell and significantly improved the cardiac function (Xie et al., 2007). In addition, overexpressing hepatocyte growth factor (HGF) modulated apoptosis of UC-MSCs and protected animals from acute liver failure (Tang et al., 2016). Also, HGF overexpression enhanced the therapeutic effect of BM-MSC for hepatocirrhosis (Zhang et al., 2018). The transduction of ERBB4 into MSCs also increased apoptotic resistance *via* activating PI3K/AKT signaling pathway (Liang et al., 2015). Moreover, HIF1α transfection improved the cardiac repair efficiency of MSCs by decreasing cardiomyocytes apoptosis (Huang et al., 2014). Importantly, the genetic upregulation of several pro-survival factors, including Bcl-2, Bcl-xl and Akt1, could increase the long-term survival of transplanted human NSCs (Korshunova et al., 2020).

## 3D Cell Culturing

Cell culture is conventionally conducted by a two-dimensional (2D) system that often does not adequately replicate the three-dimensional (3D) environment, and it is deficient in cell-to-cell interactions (Madl et al., 2018; Seo et al., 2019). The 3D culturing of bone marrow MSCs using a 3D-dynamic system exhibited decreased apoptosis and improved therapeutic effect for cardiac function (Wang Y. et al., 2018). A recently developed 3D culture clump of MSCs/extracellular matrix complexes also showed resistance against apoptosis (Komatsu et al., 2020). Furthermore, the presence of D-serine and retinoic acid in the 3D organ culture of spermatogonial SCs enhanced its therapeutic effect on spermatogenesis *via* suppressing apoptotic signaling (Modirshanechi et al., 2020). Moreover, exosomes derived from UC-MSCs under 3D culturing exerted improved osteochondral regeneration activity (Yan and Wu, 2020).

## Co-Transplantation

Co-transplantation of SC with other SCs or adult cells can also restore SCs *via* suppressing PCD. Co-transplantation of NSCs with olfactory ensheathing cells (OECs) attenuated neuronal apoptosis in traumatic brain injury (Liu et al., 2014). Also, the co-transplantation of OECs with epidermal neural crest SCs exerted a beneficial effect upon peripheral nerve injury (Zhang L. et al., 2019). Regarding repairing facial nerve axotomy, the co-transplantation of BM-MSCs and monocytes reduced apoptosis of facial nerve nucleus (Wu L. et al., 2020). Moreover, co-transplantation of ADSCs and stromal vascular fractions improved parathyroid transplantation survival *in vitro* and *in vivo* for treating hypoparathyroidism (Cui et al., 2020).

## Studies on Promoting SC Survival for Diabetes and Diabetes-Related Diseases

Regarding treating diabetes and diabetes-related diseases, multiple strategies have also been applied to improve cell survival after SC transplantation. It was reported that the hypoxic preconditioning of BM-MSCs upregulated the anti-apoptotic protein Bcl-2, thus promoting endothelial cell proliferation and decreasing the apoptosis of endothelial cells in diabetic rats (Liu et al., 2015b). Also, exposure to short-term hypoxia enhanced islet protective potential of adipose-derived MSCs (AD-MSCs) (Schive et al., 2017). In

addition, hypoxia pretreatment promoted the AD-MSCs based repair of diabetic erectile dysfunction by increasing the survival of transplanted SCs in host tissues and their expression of regenerative factors (Wang et al., 2015).

Preconditioning with pharmacological or chemical agents has promoted SCs survival for treating diabetes. Preconditioning with resveratrol significantly enhanced the viability and therapeutic effect of ADSC and increased expression of the survival marker *p*-Akt for the treatment of damaged pancreas and liver dysfunction in diabetic rats (Chen et al., 2019a; Chen et al., 2019b). In addition, pretreatment with mitoTEMPO, a mitochondrial ROS scavenger, improved the survival of ADSC in diabetic mice and decreased the limb injury (Lian et al., 2019). Moreover, treatment of MSCs in combination with melatonin decreased the rate of islet cell apoptosis *via* suppressing apoptotic signaling (Kadry et al., 2018). Also, melatonin preconditioning enhanced the effect of MSCs-derived exosomes on diabetic wound healing by regulating macrophages and targeting the PTEN/AKT pathway (Liu W. et al., 2020). Notably, although metformin, the most commonly used antidiabetic drug, and BM-MSCs treatment individually improve cardiac function in diabetic cardiomyopathy, metformin can reduce the efficacy of MSCs therapy for cardiac repair during diabetic cardiomyopathy by decreasing the survival of transplanted SCs (Ammar et al., 2021).

Genetic modification improved the survival of SCs for treating diabetes and diabetes-related diseases. The overexpression of HIF1α reduced ADSC apoptosis upon high glucose conditions and enhanced the therapeutic effects on diabetic wound healing (Xu et al., 2020). The preconditioning of MSCs with deferoxamine, an iron chelator, increased the stability of HIF1α protein and homing of MSCs in streptozotocin-diabetic rats (Najafi and Sharifi, 2013). Moreover, the overexpression of AURKA promoted the effect of ADSCs on wound healing in diabetic mice *via* enhancing autophagy of ADSCs and decreasing apoptosis (Yin et al., 2020).

It was also demonstrated that co-culturing and co-transplanting of BM-MSCs and islet reduced islet destruction *in vitro* and increased anti-inflammatory effects *in vivo* (Yoshimatsu et al., 2015). Another study reported that islets co-cultured with ADSC reduced apoptosis and improved glucose-stimulated insulin secretion compared with the control group (Gamble et al., 2018). Moreover, the co-transplantation of MSCs and fetal HSCs enhanced engraftment of HSCs and promoted the therapeutic effect in T1DM (Arjmand et al., 2019).

## Implications for Future Strategies to Improve SC Therapy for Diabetes and Diabetes-Related Diseases

Although the necroptosis and ferroptosis in SCs for treating diabetic diseases are rarely reported, recent studies indicated these two types of PCD are primary mechanisms of cell death in islet transplantation, suggesting the potential value of targeting necroptosis and ferroptosis for SC therapy (Zhao et al., 2015; Yao et al., 2020). As mentioned in this review, interactions between different types of PCD also need further study and novel regulators, such as AMPK/mTOR, which coordinate multiple cell death are promising therapeutic targets to improve SC therapy for diabetes and diabetes-related diseases (Zhang et al., 2016).

Although genetic modification is an efficient method to target PCD, the use of genetic techniques raises some safety concerns (Hu C. et al., 2020). Preconditioning strategies of SCs for transplantation are an attractive alternative to overcome this potential limitation. Increased oxidative stress is considered a major factor to compromise MSCs in diabets models (Fijany et al., 2019). Evidence supporting the benefit of acute preconditioning of SCs with oxidative stress suggests that the application of preconditioning may reduce oxidative stress-induced PCD in diabetic diseases (Pendergrass et al., 2013). Moreover, preconditioning with melatonin showed a significant protective effect on SCs *via* targeting multiple types of PCD, and melatonin suppressed osteoblasts ferroptosis induced by high glucose in type 2 diabetic osteoporosis (Liu W. et al., 2020; Zhao et al., 2020b; Ma et al., 2020). Thus, melatonin is a promising agent to improve SC survival in transplantation for treating diabetes and diabetes-related diseases.

As 3D culture systems become more relevant to innate structure and physiology, the ability to adequately replicate the 3D environment experienced by transplanted SCs becomes possible. Studies of 3D cell culturing developed to reduce PCD of SCs and improve their therapeutic effects have rapidly advanced (Shimony et al., 2008; Modirshanechi et al., 2020). In addition, a 3D capacitance cell sensor has been developed to monitor cell apoptosis in real-time for 3D cell cultures (Lee et al., 2016). Although methods of 3D cell culturing of SCs that can be used for treating diabetic diseases have been established, the application of these methods and their benefit requires further exploration.

# CONCLUSION

Extensive and increasing evidence demonstrates that distinct types of PCD contribute to the cell death of SCs, and the inhibition of PCDs can promote the survival of SCs and their therapeutic effects in diabetes and diabetes-related diseases. These findings provide deep insights into the cell death of SCs-based therapy for diabetes and diabetes-related diseases and shed light on the future development of therapeutic strategies.

# AUTHOR CONTRIBUTIONS

QZ wrote the first draft of the manuscript. XW, XH, WZ, XB, YH contributed to manuscript draft review and modification. KX and

QZ developed the idea. KX and WY supervised the work. All authors contributed to the article and approved the submitted version.

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## REFERENCES

Ammar, H. I., Shamseldeen, A. M., Shoukry, H. S., Ashour, H., Kamar, S. S., Rashed, L. A., et al. (2021). Metformin Impairs Homing Ability and Efficacy of Mesenchymal Stem Cells for Cardiac Repair in Streptozotocin-Induced Diabetic Cardiomyopathy in Rats. *Am. J. Physiology-Heart Circulatory Physiol.* 320, H1290–H1302. doi:10.1152/ajpheart.00317.2020

Andrade-Tomaz, M., De Souza, I., Rocha, C. R. R., and Gomes, L. R. (2020). The Role of Chaperone-Mediated Autophagy in Cell Cycle Control and its Implications in Cancer. *Cells* 9, 2140. doi:10.3390/cells9092140

Arjmand, B., Goodarzi, P., Aghayan, H. R., Payab, M., Rahim, F., Alavi-Moghadam, S., et al. (2019). Co-transplantation of Human Fetal Mesenchymal and Hematopoietic Stem Cells in Type 1 Diabetic Mice Model. *Front. Endocrinol.* 10, 761. doi:10.3389/fendo.2019.00761

Bayat, A.-H., Saeidikhoo, S., Ebrahimi, V., Mesgar, S., Joneidi, M., Soltani, R., et al. (2021). Bilateral Striatal Transplantation of Human Olfactory Stem Cells Ameliorates Motor Function, Prevents Necroptosis-Induced Cell Death and Improves Striatal Volume in the Rat Model of Huntington's Disease. *J. Chem. Neuroanat.* 112, 101903. doi:10.1016/j.jchemneu.2020.101903

Bedoui, S., Herold, M. J., and Strasser, A. (2020). Emerging Connectivity of Programmed Cell Death Pathways and its Physiological Implications. *Nat. Rev. Mol. Cel Biol.* 21, 678–695. doi:10.1038/s41580-020-0270-8

Beldi, G., Bahiraii, S., Lezin, C., Nouri Barkestani, M., Abdelgawad, M. E., Uzan, G., et al. (2020a). TNFR2 Is a Crucial Hub Controlling Mesenchymal Stem Cell Biological and Functional Properties. *Front. Cel Dev. Biol.* 8, 1408. doi:10.3389/fcell.2020.596831

Beldi, G., Khosravi, M., Abdelgawad, M. E., Salomon, B. L., Uzan, G., Haouas, H., et al. (2020b). TNFα/TNFR2 Signaling Pathway: an Active Immune Checkpoint for Mesenchymal Stem Cell Immunoregulatory Function. *Stem Cel. Res. Ther.* 11, 281. doi:10.1186/s13287-020-01740-5

Bialik, S., and Kimchi, A. (2010). Lethal Weapons: DAP-Kinase, Autophagy and Cell Death. *Curr. Opin. Cel Biol.* 22, 199–205. doi:10.1016/j.ceb.2009.11.004

Biswas, A., and Hutchins, R. (2007). Embryonic Stem Cells. *Stem Cell Dev.* 16, 213–222. doi:10.1089/scd.2006.0081

Bogliotti, Y. S., Wu, J., Vilarino, M., Okamura, D., Soto, D. A., Zhong, C., et al. (2018). Efficient Derivation of Stable Primed Pluripotent Embryonic Stem Cells from Bovine Blastocysts. *Proc. Natl. Acad. Sci. USA* 115, 2090–2095. doi:10.1073/pnas.1716161115

Carneiro, B. A., and El-Deiry, W. S. (2020). Targeting Apoptosis in Cancer Therapy. *Nat. Rev. Clin. Oncol.* 17, 395–417. doi:10.1038/s41571-020-0341-y

Chen, M.-T., Zhao, Y.-T., Zhou, L.-Y., Li, M., Zhang, Q., Han, Q., et al. (2021a). Exosomes Derived from Human Umbilical Cord Mesenchymal Stem Cells Enhance Insulin Sensitivity in Insulin Resistant Human Adipocytes. *Curr. Med. Sci.* 41, 87–93. doi:10.1007/s11596-021-2323-4

Chen, N.-N., Wei, F., Wang, L., Cui, S., Wan, Y., and Liu, S. (2016). Tumor Necrosis Factor Alpha Induces Neural Stem Cell Apoptosis through Activating P38 MAPK Pathway. *Neurochem. Res.* 41, 3052–3062. doi:10.1007/s11064-016-2024-8

Chen, S., Du, K., and Zou, C. (2020). Current Progress in Stem Cell Therapy for Type 1 Diabetes Mellitus. *Stem Cel. Res. Ther.* 11, 275. doi:10.1186/s13287-020-01793-6

Chen, T. S., Ju, D. T., Day, C. H., Yeh, Y. L., Chen, R. J., Viswanadha, V. P., et al. (2019a). Protective Effect of Autologous Transplantation of Resveratrol Preconditioned Adipose-derived Stem Cells in the Treatment of Diabetic Liver Dysfunction in Rat Model. *J. Tissue Eng. Regen. Med.* 13, 1629–1640. doi:10.1002/term.2917

Chen, T. S., Kuo, C. H., Day, C. H., Pan, L. F., Chen, R. J., Chen, B. C., et al. (2019b). Resveratrol Increases Stem Cell Function in the Treatment of Damaged Pancreas. *J. Cel. Physiol.* 234, 20443–20452. doi:10.1002/jcp.28646

Chen, X., Wang, Q., Li, X., Wang, Q., Xie, J., and Fu, X. (2018). Heat Shock Pretreatment of Mesenchymal Stem Cells for Inhibiting the Apoptosis of Ovarian Granulosa Cells Enhanced the Repair Effect on Chemotherapy-Induced Premature Ovarian Failure. *Stem Cel. Res. Ther.* 9, 240. doi:10.1186/s13287-018-0964-4

Chen, Y., Li, Y., Guo, L., Hong, J., Zhao, W., Hu, X., et al. (2021b). Bibliometric Analysis of the Inflammasome and Pyroptosis in Brain. *Front. Pharmacol.* 11, 2315. doi:10.3389/fphar.2020.626502

Cheng, S. Y., Wang, S. C., Lei, M., Wang, Z., and Xiong, K. (2018). Regulatory Role of Calpain in Neuronal Death. *Neural Regen. Res.* 13, 556–562. doi:10.4103/1673-5374.228762

Cho, K. Y., Nakamura, A., Omori, K., Takase, T., Miya, A., Manda, N., et al. (2019). Effect of Switching from Pioglitazone to the Sodium Glucose Co-transporter-2 Inhibitor Dapagliflozin on Body Weight and Metabolism-Related Factors in Patients with Type 2 Diabetes Mellitus: An Open-Label, Prospective, Randomized, Parallel-Group Comparison. *Diabetes Obes. Metab.* 21, 710–714. doi:10.1111/dom.13557

Chu, J., Liu, C. X., Song, R., and Li, Q. L. (2020). Ferrostatin-1 Protects HT-22 Cells from Oxidative Toxicity. *Neural Regen. Res.* 15, 528–536. doi:10.4103/1673-5374.266060

Cozzi, A., Orellana, D. I., Santambrogio, P., Rubio, A., Cancellieri, C., Giannelli, S., et al. (2019). Stem Cell Modeling of Neuroferritinopathy Reveals Iron as a Determinant of Senescence and Ferroptosis during Neuronal Aging. *Stem Cel Rep.* 13, 832–846. doi:10.1016/j.stemcr.2019.09.002

Cui, Q., Zhang, D., Kong, D., Tang, J., Liao, X., Yang, Q., et al. (2020). Co-transplantation with Adipose-Derived Cells to Improve Parathyroid Transplantation in a Mice Model. *Stem Cel. Res. Ther.* 11, 200. doi:10.1186/s13287-020-01733-4

Dou, J., Su, P., Xu, C., Wen, Z., Mao, Z., and Li, W. (2020). Targeting Hsc70-Based Autophagy to Eliminate Amyloid β Oligomers. *Biochem. Biophysical Res. Commun.* 524, 923–928. doi:10.1016/j.bbrc.2020.02.016

Fatima, A., Rrez-Garcia, R., and Vilchez, D. (2019). Guti, #233Induced Pluripotent Stem Cells from Huntington's Disease Patients: a Promising Approach to Define and Correct Disease-Related Alterations. *Neural Regen. Res.* 14, 769–770.

Ferreira, J., Teixeira, G. Q., Teixeira, G., Neto, e., Ribeiro-Machado, c., Silva, A., et al. (2021). IL-1β-pre-conditioned Mesenchymal Stem/stromal Cells' Secretome Modulates the Inflammatory Response and Aggrecan Deposition in Intervertebral Disc. *eCM* 41, 431–543. doi:10.22203/ecm.v041a28

Fijany, A., Sayadi, L. R., Khoshab, N., Banyard, D. A., Shaterian, A., Alexander, M., et al. (2019). Mesenchymal Stem Cell Dysfunction in Diabetes. *Mol. Biol. Rep.* 46, 1459–1475. doi:10.1007/s11033-018-4516-x

Font-Belmonte, E. (2020). Gonz, #225, Lez-Rodr, #237, Guez, P., Fern, #225, Ndez-L, #243, and Pez, ANecroptosis in Global Cerebral Ischemia: a Role for Endoplasmic Reticulum Stress. *Neural Regen. Res.* 15, 455–456.

Fu, X., Wu, S., Li, B., Xu, Y., and Liu, J. (2020). Functions of P53 in Pluripotent Stem Cells. *Protein Cell* 11, 71–78. doi:10.1007/s13238-019-00665-x

Gamble, A., Pawlick, R., Pepper, A. R., Bruni, A., Adesida, A., Senior, P. A., et al. (2018). Improved Islet Recovery and Efficacy through Co-culture and Co-transplantation of Islets with Human Adipose-Derived Mesenchymal Stem Cells. *PLoS One* 13, e0206449. doi:10.1371/journal.pone.0206449

Glick, D., Barth, S., and Macleod, K. F. (2010). Autophagy: Cellular and Molecular Mechanisms. *J. Pathol.* 221, 3–12. doi:10.1002/path.2697

Gong, J., Gu, H., Zhao, L., Wang, L., Liu, P., Wang, F., et al. (2018). Phosphorylation of ULK1 by AMPK Is Essential for Mouse Embryonic Stem Cell Self-Renewal and Pluripotency. *Cell Death Dis* 9, 38. doi:10.1038/s41419-017-0054-z

Gu, H., Shi, X., Liu, C., Wang, C., Sui, N., Zhao, Y., et al. (2019). USP8 Maintains Embryonic Stem Cell Stemness via Deubiquitination of EPG5. *Nat. Commun.* 10, 1465. doi:10.1038/s41467-019-09430-4

Guo, L., Du, J., Yuan, D.-F., Zhang, Y., Zhang, S., Zhang, H.-C., et al. (2020b). Optimal H2O2 Preconditioning to Improve Bone Marrow Mesenchymal Stem Cells' Engraftment in Wound Healing. *Stem Cel. Res. Ther.* 11, 434. doi:10.1186/s13287-020-01910-5

Guo, L. M., Wang, Z., Li, S. P., Wang, M., Yan, W. T., Liu, F. X., et al. (2020a). RIP3/MLKL-mediated Neuronal Necroptosis Induced by Methamphetamine at 39°C. *Neural Regen. Res.* 15, 865–874. doi:10.4103/1673-5374.268902

Gurusamy, N., Alsayari, A., Rajasingh, S., and Rajasingh, J. (2018). Adult Stem Cells for Regenerative Therapy. *Prog. Mol. Biol. Transl. Sci.* 160, 1–22. doi:10.1016/bs.pmbts.2018.07.009

Hafner, A., Bulyk, M. L., Jambhekar, A., and Lahav, G. (2019). The Multiple Mechanisms that Regulate P53 Activity and Cell Fate. *Nat. Rev. Mol. Cel Biol.* 20, 199–210. doi:10.1038/s41580-019-0110-x

Hajmousa, G., Elorza, A. A., Nies, V. J. M., Jensen, E. L., Nagy, R. A., and Harmsen, M. C. (2016). Hyperglycemia Induces Bioenergetic Changes in Adipose-Derived Stromal Cells while Their Pericytic Function Is Retained. *Stem Cell Dev.* 25, 1444–1453. doi:10.1089/scd.2016.0025

He, Q., Wang, L., Zhao, R., Yan, F., Sha, S., Cui, C., et al. (2020a). Mesenchymal Stem Cell-Derived Exosomes Exert Ameliorative Effects in Type 2 Diabetes by Improving Hepatic Glucose and Lipid Metabolism via Enhancing Autophagy. *Stem Cel. Res. Ther.* 11, 223. doi:10.1186/s13287-020-01731-6

He, Z., An, S., Chen, J., Zhang, S., Tan, C., Yu, J., et al. (2020b). Neural Progenitor Cell Pyroptosis Contributes to Zika Virus-Induced Brain Atrophy and Represents a Therapeutic Target. *Proc. Natl. Acad. Sci. USA* 117, 23869–23878. doi:10.1073/pnas.2007773117

Ho, T. T., Warr, M. R., Adelman, E. R., Lansinger, O. M., Flach, J., Verovskaya, E. V., et al. (2017). Autophagy Maintains the Metabolism and Function of Young and Old Stem Cells. *Nature* 543, 205–210. doi:10.1038/nature21388

Hu, B., Zhang, S., Liu, W., Wang, P., Chen, S., Lv, X., et al. (2020a). Inhibiting Heat Shock Protein 90 Protects Nucleus Pulposus-Derived Stem/Progenitor Cells from Compression-Induced Necroptosis and Apoptosis. *Front Cel Dev Biol* 8, 685. doi:10.3389/fcell.2020.00685

Hu, C., and Li, L. (2018). Preconditioning Influences Mesenchymal Stem Cell Properties *In Vitro* and *In Vivo*. *J. Cel. Mol. Med.* 22, 1428–1442. doi:10.1111/jcmm.13492

Hu, C., Wu, Z., and Li, L. (2020b). Pre-treatments Enhance the Therapeutic Effects of Mesenchymal Stem Cells in Liver Diseases. *J. Cel. Mol. Med.* 24, 40–49. doi:10.1111/jcmm.14788

Hu, X.-M., Zhang, Q., Zhou, R.-X., Wu, Y.-L., Li, Z.-X., Zhang, D.-Y., et al. (2021). Programmed Cell Death in Stem Cell-Based Therapy: Mechanisms and Clinical Applications. *Wjsc* 13, 386–415. doi:10.4252/wjsc.v13.i5.386

Hu, Y., Huang, L., Shen, M., Liu, Y., Liu, G., Wu, Y., et al. (2019). Pioglitazone Protects Compression-Mediated Apoptosis in Nucleus Pulposus Mesenchymal Stem Cells by Suppressing Oxidative Stress. *Oxid. Med. Cel. Longev.* 2019, 4764071. doi:10.1155/2019/4764071

Huang, B., Qian, J., Ma, J., Huang, Z., Shen, Y., Chen, X., et al. (2014). Myocardial Transfection of Hypoxia-Inducible Factor-1α and Co-transplantation of Mesenchymal Stem Cells Enhance Cardiac Repair in Rats with Experimental Myocardial Infarction. *Stem Cel. Res. Ther.* 5, 22. doi:10.1186/scrt410

Huang, J., Zhang, Y., Bersenev, A., O'Brien, W. T., Tong, W., Emerson, S. G., et al. (2009). Pivotal Role for Glycogen Synthase Kinase-3 in Hematopoietic Stem Cell Homeostasis in Mice. *J. Clin. Invest.* 119, 3519–3529. doi:10.1172/JCI40572

Huang, Q., Ding, Y., Yu, J.-G., Li, J., Xiang, Y., and Tao, N. (2021a). Induction of Differentiation of Mesenchymal Stem Cells into Retinal Pigment Epithelial Cells for Retinal Regeneration by Using Ciliary Neurotrophic Factor in Diabetic Rats. *Curr. Med. Sci.* 41, 145–152. doi:10.1007/s11596-021-2329-y

Huang, Y., Wang, S., Huang, F., Zhang, Q., Qin, B., Liao, L., et al. (2021b). c-FLIP Regulates Pyroptosis in Retinal Neurons Following Oxygen-Glucose Deprivation/recovery via a GSDMD-Mediated Pathway. *Ann. Anat. - Anatomischer Anzeiger* 235, 151672. doi:10.1016/j.aanat.2020.151672

Ishida, T., Suzuki, S., Lai, C. Y., Yamazaki, S., Kakuta, S., Iwakura, Y., et al. (2017). Pre-Transplantation Blockade of TNF-α-Mediated Oxygen Species Accumulation Protects Hematopoietic Stem Cells. *Stem Cells* 35, 989–1002. doi:10.1002/stem.2524

Jin, P., Zhang, X., Wu, Y., Li, L., Yin, Q., Zheng, L., et al. (2010). Streptozotocin-Induced Diabetic Rat-Derived Bone Marrow Mesenchymal Stem Cells Have Impaired Abilities in Proliferation, Paracrine, Antiapoptosis, and Myogenic Differentiation. *Transplant. Proc.* 42, 2745–2752. doi:10.1016/j.transproceed.2010.05.145

Kadry, S. M., El-Dakdoky, M. H., Haggag, N. Z., Rashed, L. A., and Hassen, M. T. (2018). Melatonin Improves the Therapeutic Role of Mesenchymal Stem Cells in Diabetic Rats. *Toxicol. Mech. Methods* 28, 529–538. doi:10.1080/15376516.2018.1471634

Kim, J., Kundu, M., Viollet, B., and Guan, K.-L. (2011). AMPK and mTOR Regulate Autophagy through Direct Phosphorylation of Ulk1. *Nat. Cel Biol.* 13, 132–141. doi:10.1038/ncb2152

Komatsu, N., Kajiya, M., Morimoto, S., Motoike, S., Yoshii, H., Iwata, T., et al. (2020). Cox2-mediated PGE2 Production via p38/JNK-C-Fos Signaling Inhibits Cell Apoptosis in 3D Floating Culture Clumps of Mesenchymal Stem Cell/extracellular Matrix Complexes. *Biochem. Biophysical Res. Commun.* 530, 448–454. doi:10.1016/j.bbrc.2020.05.100

Kong, Y., Cheng, L., Ma, L., Li, H., Cheng, B., and Zhao, Y. (2019). Norepinephrine Protects against Apoptosis of Mesenchymal Stem Cells Induced by High Glucose. *J. Cel. Physiol.* 234, 20801–20815. doi:10.1002/jcp.28686

Korshunova, I., Rhein, S., García-González, D., Stölting, I., Pfisterer, U., Barta, A., et al. (2020). Genetic Modification Increases the Survival and the Neuroregenerative Properties of Transplanted Neural Stem Cells. *JCI Insight* 5. doi:10.1172/jci.insight.126268

Lake, B. B., Fink, J., Klemetsaune, L., Fu, X., Jeffers, J. R., Zambetti, G. P., et al. (2012). Context-Dependent Enhancement of Induced Pluripotent Stem Cell Reprogramming by Silencing Puma. *Stem Cells* 30, 888–897. doi:10.1002/stem.1054

Lan, Y.-W., Choo, K.-B., Chen, C.-M., Hung, T.-H., Chen, Y.-B., Hsieh, C.-H., et al. (2015). Hypoxia-preconditioned Mesenchymal Stem Cells Attenuate Bleomycin-Induced Pulmonary Fibrosis. *Stem Cel. Res. Ther.* 6, 97. doi:10.1186/s13287-015-0081-6

Lee, S.-M., Han, N., Lee, R., Choi, I.-H., Park, Y.-B., Shin, J.-S., et al. (2016). Real-time Monitoring of 3D Cell Culture Using a 3D Capacitance Biosensor. *Biosens. Bioelectron.* 77, 56–61. doi:10.1016/j.bios.2015.09.005

Leitch, H. A., Fibach, E., and Rachmilewitz, E. (2017). Toxicity of Iron Overload and Iron Overload Reduction in the Setting of Hematopoietic Stem Cell Transplantation for Hematologic Malignancies. *Crit. Rev. Oncology/Hematology* 113, 156–170. doi:10.1016/j.critrevonc.2017.03.002

Li, Q., Yin, Y., Zheng, Y., Chen, F., and Jin, P. (2018). Inhibition of Autophagy Promoted High glucose/ROS-Mediated Apoptosis in ADSCs. *Stem Cel. Res. Ther.* 9, 289. doi:10.1186/s13287-018-1029-4

Li, X., Zeng, J., Liu, Y., Liang, M., Liu, Q., Li, Z., et al. (2020). Inhibitory Effect and Mechanism of Action of Quercetin and Quercetin Diels-Alder Anti-dimer on Erastin-Induced Ferroptosis in Bone Marrow-Derived Mesenchymal Stem Cells. *Antioxidants* 9, 205. doi:10.3390/antiox9030205

Lian, K., Wang, Q., Zhao, S., Yang, M., Chen, G., Chen, Y., et al. (2019). Pretreatment of Diabetic Adipose-Derived Stem Cells with mitoTEMPO Reverses Their Defective Proangiogenic Function in Diabetic Mice with Critical Limb Ischemia. *Cel Transpl.* 28, 1652–1663. doi:10.1177/0963689719885076

Liang, X., Ding, Y., Zhang, Y., Chai, Y.-H., He, J., Chiu, S.-M., et al. (2015). Activation of NRG1-ERBB4 Signaling Potentiates Mesenchymal Stem Cell-Mediated Myocardial Repairs Following Myocardial Infarction. *Cel Death Dis* 6, e1765. doi:10.1038/cddis.2015.91

Liao, L.-S., Lu, S., Yan, W.-T., Wang, S.-C., Guo, L.-M., Yang, Y.-D., et al. (2021). The Role of HSP90α in Methamphetamine/Hyperthermia-Induced Necroptosis in Rat Striatal Neurons. *Front. Pharmacol.* 12, 716394. doi:10.3389/fphar.2021.716394

Liu, J., Hao, H., Huang, H., Tong, C., Ti, D., Dong, L., et al. (2015a). Hypoxia Regulates the Therapeutic Potential of Mesenchymal Stem Cells through Enhanced Autophagy. *The Int. J. Lower Extremity Wounds* 14, 63–72. doi:10.1177/1534734615573660

Liu, J., Hao, H., Xia, L., Ti, D., Huang, H., Dong, L., et al. (2015b). Hypoxia Pretreatment of Bone Marrow Mesenchymal Stem Cells Facilitates Angiogenesis by Improving the Function of Endothelial Cells in Diabetic Rats with Lower Ischemia. *PLoS One* 10, e0126715. doi:10.1371/journal.pone.0126715

Liu, K., Zhao, Q., Liu, P., Cao, J., Gong, J., Wang, C., et al. (2016). ATG3-dependent Autophagy Mediates Mitochondrial Homeostasis in Pluripotency Acquirement and Maintenance. *Autophagy* 12, 2000–2008. doi:10.1080/15548627.2016.1212786

Liu, L., Gao, J., Yuan, Y., Chang, Q., Liao, Y., and Lu, F. (2013). Hypoxia Preconditioned Human Adipose Derived Mesenchymal Stem Cells Enhance Angiogenic Potential via Secretion of Increased VEGF and bFGF. *Cell Biol. Int.* 37, 551–560. doi:10.1002/cbin.10097

Liu, P., Liu, K., Gu, H., Wang, W., Gong, J., Zhu, Y., et al. (2017). High Autophagic Flux Guards ESC Identity through Coordinating Autophagy Machinery Gene Program by FOXO1. *Cell Death Differ* 24, 1672–1680. doi:10.1038/cdd.2017.90

Liu, S.-J., Zou, Y., Belegu, V., Lv, L.-Y., Lin, N., Wang, T.-Y., et al. (2014). Co-grafting of neural stem cells with olfactory en sheathing cells promotes neuronal restoration in traumatic brain injury with an anti-inflammatory mechanism. *J. Neuroinflammation* 11, 66. doi:10.1186/1742-2094-11-66

Liu, W., Yu, M., Xie, D., Wang, L., Ye, C., Zhu, Q., et al. (2020a). Melatonin-stimulated MSC-Derived Exosomes Improve Diabetic Wound Healing through Regulating Macrophage M1 and M2 Polarization by Targeting the PTEN/AKT Pathway. *Stem Cel. Res. Ther.* 11, 259. doi:10.1186/s13287-020-01756-x

Liu, Y., Li, P., Qiao, C., Wu, T., Sun, X., Wen, M., et al. (2020b). Chitosan Hydrogel Enhances the Therapeutic Efficacy of Bone Marrow-Derived Mesenchymal Stem Cells for Myocardial Infarction by Alleviating Vascular Endothelial Cell Pyroptosis. *J. Cardiovasc. Pharmacol.* 75, 75–83. doi:10.1097/fjc.0000000000000760

Lv, H., Yuan, X., Zhang, J., Lu, T., Yao, J., Zheng, J., et al. (2021). Heat Shock Preconditioning Mesenchymal Stem Cells Attenuate Acute Lung Injury via Reducing NLRP3 Inflammasome Activation in Macrophages. *Stem Cel. Res. Ther.* 12, 290. doi:10.1186/s13287-021-02328-3

Ma, H., Wang, X., Zhang, W., Li, H., Zhao, W., Sun, J., et al. (2020). Melatonin Suppresses Ferroptosis Induced by High Glucose via Activation of the Nrf2/HO-1 Signaling Pathway in Type 2 Diabetic Osteoporosis. *Oxid. Med. Cel. Longev.* 2020, 9067610. doi:10.1155/2020/9067610

Madl, C. M., Heilshorn, S. C., and Blau, H. M. (2018). Bioengineering Strategies to Accelerate Stem Cell Therapeutics. *Nature* 557, 335–342. doi:10.1038/s41586-018-0089-z

Manzar, G. S., Kim, E.-M., and Zavazava, N. (2017). Demethylation of Induced Pluripotent Stem Cells from Type 1 Diabetic Patients Enhances Differentiation into Functional Pancreatic β Cells. *J. Biol. Chem.* 292, 14066–14079. doi:10.1074/jbc.m117.784280

Mastri, M., Lin, H., and Lee, T. (2014). Enhancing the Efficacy of Mesenchymal Stem Cell Therapy. *Wjsc* 6, 82–93. doi:10.4252/wjsc.v6.i2.82

Matsuzawa-Ishimoto, Y., Shono, Y., Gomez, L. E., Hubbard-Lucey, V. M., Cammer, M., Neil, J., et al. (2017). Autophagy Protein ATG16L1 Prevents Necroptosis in the Intestinal Epithelium. *J. Exp. Med.* 214, 3687–3705. doi:10.1084/jem.20170558

Mcelhinney, J. M. W. R., Hasan, A., and Sajini, A. A. (2020). The Epitranscriptome Landscape of Small Noncoding RNAs in Stem Cells. *Stem Cells* 38, 1216–1228. doi:10.1002/stem.3233

Mias, C., Trouche, E., Seguelas, M. H., Calcagno, F., Dignat-George, F., Sabatier, F., et al. (2008). *Ex Vivo* Pretreatment with Melatonin Improves Survival, Proangiogenic/Mitogenic Activity, and Efficiency of Mesenchymal Stem Cells Injected into Ischemic Kidney. *Stem Cells* 26, 1749–1757. doi:10.1634/stemcells.2007-1000

Modirshanechi, G., Eslampour, M. A., and Abdolmaleki, Z. (2020). Agonist and Antagonist NMDA Receptor Effect on Cell Fate during Germ Cell Differentiation and Regulate Apoptotic Process in 3D Organ Culture. *Andrologia* 52, e13764. doi:10.1111/and.13764

Najafi, R., and Sharifi, A. M. (2013). Deferoxamine Preconditioning Potentiates Mesenchymal Stem Cell Homingin Vitroand in Streptozotocin-Diabetic Rats. *Expert Opin. Biol. Ther.* 13, 959–972. doi:10.1517/14712598.2013.782390

Naserian, S., Abdelgawad, M. E., Afshar Bakshloo, M., Ha, G., Arouche, N., Cohen, J. L., et al. (2020). The TNF/TNFR2 Signaling Pathway Is a Key Regulatory Factor in Endothelial Progenitor Cell Immunosuppressive Effect. *Cell Commun Signal* 18, 94. doi:10.1186/s12964-020-00564-3

Nikoletopoulou, V., Markaki, M., Palikaras, K., and Tavernarakis, N. (2013). Crosstalk between Apoptosis, Necrosis and Autophagy. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1833, 3448–3459. doi:10.1016/j.bbamcr.2013.06.001

Nobuta, H., Yang, N., Ng, Y. H., Marro, S. G., Sabeur, K., Chavali, M., et al. (2019). Oligodendrocyte Death in Pelizaeus-Merzbacher Disease Is Rescued by Iron Chelation. *Cell Stem Cell* 25, 531–541. doi:10.1016/j.stem.2019.09.003

Noiseux, N., Borie, M., Desnoyers, A., Menaouar, A., Stevens, L. M., Mansour, S., et al. (2012). Preconditioning of Stem Cells by Oxytocin to Improve Their Therapeutic Potential. *Endocrinology* 153, 5361–5372. doi:10.1210/en.2012-1402

Nouri Barkestani, M., Shamdani, S., Afshar Bakshloo, M., Arouche, N., Bambai, B., Uzan, G., et al. (2021). TNFα Priming through its Interaction with TNFR2 Enhances Endothelial Progenitor Cell Immunosuppressive Effect: new hope for Their Widespread Clinical Application. *Cel Commun Signal* 19, 1. doi:10.1186/s12964-020-00683-x

Pendergrass, K. D., Boopathy, A. V., Seshadri, G., Maiellaro-Rafferty, K., Che, P. L., Brown, M. E., et al. (2013). Acute Preconditioning of Cardiac Progenitor Cells with Hydrogen Peroxide Enhances Angiogenic Pathways Following Ischemia-Reperfusion Injury. *Stem Cell Dev.* 22, 2414–2424. doi:10.1089/scd.2012.0673

Pierozan, P., Cattani, D., and Karlsson, O. (2020). Hippocampal Neural Stem Cells Are More Susceptible to the Neurotoxin BMAA Than Primary Neurons: Effects on Apoptosis, Cellular Differentiation, Neurite Outgrowth, and DNA Methylation. *Cel Death Dis* 11, 910. doi:10.1038/s41419-020-03093-6

Qin, B., Zhang, Q., Chen, D., Yu, H. Y., Luo, A. X., Suo, L. P., et al. (2021). Extracellular Vesicles Derived from Mesenchymal Stem Cells: A Platform that Can Be Engineered. *Histol. Histopathol.* 36, 615–632. doi:10.14670/HH-18-297

Razazian, M., Khosravi, M., Bahiraii, S., Uzan, G., Shamdani, S., and Naserian, S. (2021). Differences and Similarities between Mesenchymal Stem Cell and Endothelial Progenitor Cell Immunoregulatory Properties against T Cells. *Wjsc* 13, 971–984. doi:10.4252/wjsc.v13.i8.971

Saleem, M., Sabir, S., Akhtar, M. F., Zahid, S., Niazi, S. G., Naeem, M., et al. (2019). Stem Cell Therapy for Diabetes Mellitus: Recent Progress and Hurdles. *Crit. Rev. Eukaryot. Gene Expr.* 29, 471–482. doi:10.1615/critreveukaryotgeneexpr.2019025723

Sart, S., Ma, T., and Li, Y. (2014). Preconditioning Stem Cells for *In Vivo* Delivery. *BioResearch Open Access* 3, 137–149. doi:10.1089/biores.2014.0012

Schive, S. W., Mirlashari, M. R., Hasvold, G., Wang, M., Josefsen, D., Gullestad, H. P., et al. (2017). Human Adipose-Derived Mesenchymal Stem Cells Respond to Short-Term Hypoxia by Secreting Factors Beneficial for Human Islets *In Vitro* and Potentiate Antidiabetic Effect *In Vivo*. *Cell Med* 9, 103–116. doi:10.3727/215517917x693401

Seo, Y., Shin, T.-H., and Kim, H.-S. (2019). Current Strategies to Enhance Adipose Stem Cell Function: An Update. *Ijms* 20, 3827. doi:10.3390/ijms20153827

Shimony, N., Avrahami, I., Gorodetsky, R., Elkin, G., Tzukert, K., Zangi, L., et al. (2008). A 3D Rotary Renal and Mesenchymal Stem Cell Culture Model Unveils Cell Death Mechanisms Induced by Matrix Deficiency and Low Shear Stress. *Nephrol. Dial. Transplant.* 23, 2071–2080. doi:10.1093/ndt/gfn062

Song, W.-J., Li, Q., Ryu, M.-O., Nam, A., An, J.-H., Jung, Y. C., et al. (2019). Canine Adipose Tissue-Derived Mesenchymal Stem Cells Pre-treated with TNF-Alpha Enhance Immunomodulatory Effects in Inflammatory Bowel Disease in Mice. *Res. Vet. Sci.* 125, 176–184. doi:10.1016/j.rvsc.2019.06.012

Stockwell, B. R., Friedmann Angeli, J. P., Bayir, H., Bush, A. I., Conrad, M., Dixon, S. J., et al. (2017). Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* 171, 273–285. doi:10.1016/j.cell.2017.09.021

Sun, X., Fang, B., Zhao, X., Zhang, G., and Ma, H. (2014). Preconditioning of Mesenchymal Stem Cells by Sevoflurane to Improve Their Therapeutic Potential. *PLoS One* 9, e90667. doi:10.1371/journal.pone.0090667

Suvorova, I. I., Knyazeva, A. R., Petukhov, A. V., Aksenov, N. D., and Pospelov, V. A. (2019). Resveratrol Enhances Pluripotency of Mouse Embryonic Stem Cells by Activating AMPK/Ulk1 Pathway. *Cel Death Discov.* 5, 61. doi:10.1038/s41420-019-0137-y

Tang, Y., Li, Q., Meng, F., Huang, X., Li, C., Zhou, X., et al. (20162016). Therapeutic Potential of HGF-Expressing Human Umbilical Cord Mesenchymal Stem Cells in Mice with Acute Liver Failure. *Int. J. Hepatol.* 2016, 5452487. doi:10.1155/2016/5452487

Vázquez, P., Arroba, A. I., Cecconi, F., De La Rosa, E. J., Boya, P., and De Pablo, F. (2012). Atg5 and Ambra1 Differentially Modulate Neurogenesis in Neural Stem Cells. *Autophagy* 8, 187–199. doi:10.4161/auto.8.2.18535

Wang, J., Li, Z., Zhang, Y., Liu, X., Chen, L., and Chen, Y. (2013a). CX43 Change in LPS Preconditioning against Apoptosis of Mesenchymal Stem Cells Induced by Hypoxia and Serum Deprivation Is Associated with ERK Signaling Pathway. *Mol. Cel. Biochem.* 380, 267–275. doi:10.1007/s11010-013-1683-x

Wang, M., Liang, X., Cheng, M., Yang, L., Liu, H., Wang, X., et al. (2019). Homocysteine Enhances Neural Stem Cell Autophagy in *In Vivo* and *In Vitro* Model of Ischemic Stroke. *Cel Death Dis* 10, 561. doi:10.1038/s41419-019-1798-4

Wang, M., Wan, H., Wang, S., Liao, L., Huang, Y., Guo, L., et al. (2020a). RSK3 Mediates Necroptosis by Regulating Phosphorylation of RIP3 in Rat Retinal Ganglion Cells. *J. Anat.* 237, 29–47. doi:10.1111/joa.13185

Wang, R., Li, H., Wu, J., Cai, Z.-Y., Li, B., Ni, H., et al. (2020b). Gut Stem Cell Necroptosis by Genome Instability Triggers Bowel Inflammation. *Nature* 580, 386–390. doi:10.1038/s41586-020-2127-x

Wang, S., Xia, P., Ye, B., Huang, G., Liu, J., and Fan, Z. (2013b). Transient Activation of Autophagy via Sox2-Mediated Suppression of mTOR Is an Important Early Step in Reprogramming to Pluripotency. *Cell Stem Cell* 13, 617–625. doi:10.1016/j.stem.2013.10.005

Wang, X., Liu, C., Li, S., Xu, Y., Chen, P., Liu, Y., et al. (2015). Hypoxia Precondition Promotes Adipose-Derived Mesenchymal Stem Cells Based Repair of Diabetic Erectile Dysfunction via Augmenting Angiogenesis and Neuroprotection. *PLoS One* 10, e0118951. doi:10.1371/journal.pone.0118951

Wang, Y., Zhang, J., Qin, Z., Fan, Z., Lu, C., Chen, B., et al. (2018a). Preparation of High Bioactivity Multilayered Bone-Marrow Mesenchymal Stem Cell Sheets for Myocardial Infarction Using a 3D-Dynamic System. *Acta Biomater.* 72, 182–195. doi:10.1016/j.actbio.2018.03.052

Wang, Z., Guo, L.-M., Wang, S.-C., Chen, D., Yan, J., Liu, F.-X., et al. (2018b). Progress in Studies of Necroptosis and its Relationship to Disease Processes. *Pathol. - Res. Pract.* 214, 1749–1757. doi:10.1016/j.prp.2018.09.002

Warr, M. R., Binnewies, M., Flach, J., Reynaud, D., Garg, T., Malhotra, R., et al. (2013). FOXO3A Directs a Protective Autophagy Program in Haematopoietic Stem Cells. *Nature* 494, 323–327. doi:10.1038/nature11895

Wu, L., Han, D., Jiang, J., Xie, X., Zhao, X., Ke, T., et al. (2020a). Co-transplantation of Bone Marrow Mesenchymal Stem Cells and Monocytes in the Brain Stem to Repair the Facial Nerve Axotomy. *Eur. J. Histochem.* 64, 3136. doi:10.4081/ejh.2020.3136

Wu, X., Hu, X., Zhang, Q., Liu, F., and Xiong, K. (2020b). Regulatory Role of Chinese Herbal Medicine in Regulated Neuronal Death. *CNS Neurol. Disord. Drug Targets* 19, 1–21. doi:10.2174/1871527319666200730165011

Xie, X., Cao, F., Sheikh, A. Y., Li, Z., Connolly, A. J., Pei, X., et al. (2007). Genetic Modification of Embryonic Stem Cells with VEGF Enhances Cell Survival and Improves Cardiac Function. *Cloning and Stem Cells* 9, 549–563. doi:10.1089/clo.2007.0032

Xie, Y., Chen, H., Luo, D., Yang, X., Yao, J., Zhang, C., et al. (2020). Inhibiting Necroptosis of Spermatogonial Stem Cell as a Novel Strategy for Male Fertility Preservation. *Stem Cell Dev.* 29, 475–487. doi:10.1089/scd.2019.0220

Xu, J., Liu, X., Zhao, F., Zhang, Y., and Wang, Z. (2020). HIF1α Overexpression Enhances Diabetic Wound Closure in High Glucose and Low Oxygen Conditions by Promoting Adipose-Derived Stem Cell Paracrine Function and Survival. *Stem Cel. Res. Ther.* 11, 148. doi:10.1186/s13287-020-01654-2

Yadav, S. K., Kambis, T. N., Kar, S., Park, S. Y., and Mishra, P. K. (2020). MMP9 Mediates Acute Hyperglycemia-Induced Human Cardiac Stem Cell Death by Upregulating Apoptosis and Pyroptosis *In Vitro*. *Cel Death Dis* 11, 186. doi:10.1038/s41419-020-2367-6

Yamashita, M., Nitta, E., and Suda, T. (2015). Aspp1 Preserves Hematopoietic Stem Cell Pool Integrity and Prevents Malignant Transformation. *Cell stem cell* 17, 23–34. doi:10.1016/j.stem.2015.05.013

Yamashita, M., Nitta, E., and Suda, T. (2016). Regulation of Hematopoietic Stem Cell Integrity through P53 and its Related Factors. *Ann. N.Y. Acad. Sci.* 1370, 45–54. doi:10.1111/nyas.12986

Yamashita, M., and Passegué, E. (2019). TNF-α Coordinates Hematopoietic Stem Cell Survival and Myeloid Regeneration. *Cell Stem Cell* 25, 357–372. doi:10.1016/j.stem.2019.05.019

Yan, B., Zhang, Y., Liang, C., Liu, B., Ding, F., Wang, Y., et al. (2020). Stem Cell-Derived Exosomes Prevent Pyroptosis and Repair Ischemic Muscle Injury through a Novel exosome/circHIPK3/FOXO3a Pathway. *Theranostics* 10, 6728–6742. doi:10.7150/thno.42259

Yan, L., and Wu, X. (2020). Exosomes Produced from 3D Cultures of Umbilical Cord Mesenchymal Stem Cells in a Hollow-Fiber Bioreactor Show Improved Osteochondral Regeneration Activity. *Cell Biol. Toxicol.* 36, 165–178. doi:10.1007/s10565-019-09504-5

Yan, W.-T., Yang, Y.-D., Hu, X.-M., Ning, W.-Y., Liao, L.-S., Lu, S., et al. (2021b). Do pyroptosis, Apoptosis, and Necroptosis (PANoptosis) Exist in Cerebral Ischemia? Evidence from Cell and Rodent Studies. *Neural Regen. Res.* 17. doi:10.4103/1673-5374.331539

Yan, W. T., Lu, S., Yang, Y. D., Ning, W. Y., Cai, Y., Hu, X. M., et al. (2021a). Research Trends, Hot Spots and Prospects for Necroptosis in the Field of Neuroscience. *Neural Regen. Res.* 16, 1628–1637. doi:10.4103/1673-5374.303032

Yang, R., Liu, F., Wang, J., Chen, X., Xie, J., and Xiong, K. (2019). Epidermal Stem Cells in Wound Healing and Their Clinical Applications. *Stem Cel. Res. Ther.* 10, 229. doi:10.1186/s13287-019-1312-z

Yang, R., Yang, S., Zhao, J., Hu, X., Chen, X., Wang, J., et al. (2020). Progress in Studies of Epidermal Stem Cells and Their Application in Skin Tissue Engineering. *Stem Cel. Res. Ther.* 11, 303. doi:10.1186/s13287-020-01796-3

Yao, Q., Sun, R., Bao, S., Chen, R., and Kou, L. (2020). Bilirubin Protects Transplanted Islets by Targeting Ferroptosis. *Front. Pharmacol.* 11, 907. doi:10.3389/fphar.2020.00907

Yin, Y., Chen, F., Li, J., Yang, J., Li, Q., and Jin, P. (2020). AURKA Enhances Autophagy of Adipose Derived Stem Cells to Promote Diabetic Wound Repair via Targeting FOXO3a. *J. Invest. Dermatol.* 140, 1639–1649. doi:10.1016/j.jid.2019.12.032

Yoshimatsu, G., Sakata, N., Tsuchiya, H., Minowa, T., Takemura, T., Morita, H., et al. (2015). The Co-transplantation of Bone Marrow Derived Mesenchymal Stem Cells Reduced Inflammation in Intramuscular Islet Transplantation. *PLoS One* 10, e0117561. doi:10.1371/journal.pone.0117561

Yuan, J., Amin, P., and Ofengeim, D. (2019). Necroptosis and RIPK1-Mediated Neuroinflammation in CNS Diseases. *Nat. Rev. Neurosci.* 20, 19–33. doi:10.1038/s41583-018-0093-1

Zhang, C., Zhao, C., Chen, X., Tao, R., Wang, S., Meng, G., et al. (2020a). Induction of ASC Pyroptosis Requires Gasdermin D or Caspase-1/11-dependent Mediators and IFNβ from Pyroptotic Macrophages. *Cel Death Dis* 11, 470. doi:10.1038/s41419-020-2664-0

Zhang, J., Zhang, J., Zhang, Y., Liu, W., Ni, W., Huang, X., et al. (2020b). Mesenchymal Stem Cells-derived Exosomes Ameliorate Intervertebral Disc Degeneration through Inhibiting Pyroptosis. *J. Cel. Mol. Med.* 24, 11742–11754. doi:10.1111/jcmm.15784

Zhang, K., Liu, F., Jin, D., Guo, T., Hou, R., Zhang, J., et al. (2019a). Autophagy Preserves the Osteogenic Ability of Periodontal Ligament Stem Cells under High Glucose Conditions in Rats. *Arch. Oral Biol.* 101, 172–179. doi:10.1016/j.archoralbio.2019.03.020

Zhang, L., Li, B., Liu, B., and Dong, Z. (2019b). Co-transplantation of Epidermal Neural Crest Stem Cells and Olfactory Ensheathing Cells Repairs Sciatic Nerve Defects in Rats. *Front. Cel. Neurosci.* 13, 253. doi:10.3389/fncel.2019.00253

Zhang, W., Wang, Y., Kong, J., Dong, M., Duan, H., and Chen, S. (2017). Therapeutic Efficacy of Neural Stem Cells Originating from Umbilical Cord-Derived Mesenchymal Stem Cells in Diabetic Retinopathy. *Sci. Rep.* 7, 408. doi:10.1038/s41598-017-00298-2

Zhang, Y., Li, R., Rong, W., Han, M., Cui, C., Feng, Z., et al. (2018). Therapeutic Effect of Hepatocyte Growth Factor-Overexpressing Bone Marrow-Derived Mesenchymal Stem Cells on CCl4-Induced Hepatocirrhosis. *Cel Death Dis* 9, 1186. doi:10.1038/s41419-018-1239-9

Zhang, Z. N., Chung, S. K., Xu, Z., and Xu, Y. (2014). Oct4 Maintains the Pluripotency of Human Embryonic Stem Cells by Inactivating P53 through Sirt1-Mediated Deacetylation. *Stem Cells* 32, 157–165. doi:10.1002/stem.1532

Zhang, Z., Yang, M., Wang, Y., Wang, L., Jin, Z., Ding, L., et al. (2016). Autophagy Regulates the Apoptosis of Bone Marrow-Derived Mesenchymal Stem Cells under Hypoxic Condition via AMP-Activated Protein Kinase/mammalian Target of Rapamycin Pathway. *Cel Biol. Int.* 40, 671–685. doi:10.1002/cbin.10604

Zhao, L., Guo, Z., Chen, K., Yang, W., Wan, X., Zeng, P., et al. (2020a). Combined Transplantation of Mesenchymal Stem Cells and Endothelial Colony-Forming Cells Accelerates Refractory Diabetic Foot Ulcer Healing. *Stem Cell Int* 2020, 8863649. doi:10.1155/2020/8863649

Zhao, L., Hu, C., Zhang, P., Jiang, H., and Chen, J. (2020b). Melatonin Preconditioning Is an Effective Strategy for Mesenchymal Stem Cell-based Therapy for Kidney Disease. *J. Cel. Mol. Med.* 24, 25–33. doi:10.1111/jcmm.14769

Zhao, Y., Scott, N. A., Fynch, S., Elkerbout, L., Wong, W. W.-L., Mason, K. D., et al. (2015). Autoreactive T Cells Induce Necrosis and Not BCL-2-Regulated or Death Receptor-Mediated Apoptosis or RIPK3-dependent Necroptosis of Transplanted Islets in a Mouse Model of Type 1 Diabetes. *Diabetologia* 58, 140–148. doi:10.1007/s00125-014-3407-5

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# CHNQD-00603 Promotes Osteogenic Differentiation of Bone Marrow Mesenchymal Stem Cells by the miR-452-3p-Mediated Autophagy Pathway

*Shanshan Xin*[1,2], *Shao-Ming Li*[1,2], *Ling Gao*[1,3], *Jing-Jing Zheng*[4], *Yan-Wei Wu*[5], *Chang-Lun Shao*[5], *Wen-Hao Ren*[1] and *Keqian Zhi*[1,2,3]*

[1]Department of Oral and Maxillofacial Surgery, The Affiliated Hospital of Qingdao University, Qingdao, China, [2]School of Stomatology, Qingdao University, Qingdao, China, [3]Key Lab of Oral Clinical Medicine, The Affiliated Hospital of Qingdao University, Qingdao, China, [4]Department of Endodontics, The Affiliated Hospital of Qingdao University, Qingdao, China, [5]Key Laboratory of Marine Drugs, The Ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao, China

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**Edited by:**
Pakpoom Kheolamai,
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***Correspondence:**
Keqian Zhi
zhikeqianqd@qdu.edu.cn
zhikeqian@sina.com

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**Background:** Periodontitis is a chronic and progressive disease accompanied by bone loss. It is still a challenge to restore the bone structure. The osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) plays a decisive role in bone restoration and regeneration. Marine natural products (MNPs) have multiple biological activities, including anti-tumor and anti-inflammatory properties. However, the exploration of MNPs in osteogenesis is far from sufficient.

**Methods:** We obtained a series of derivatives through structural optimization from 4-phenyl-3,4-dihydroquinolin-2(1H)-one alkaloid isolated from *Scopulariopsis* sp. Some preliminary cytological experiments showed that CHNQD-00603, obtained by adding a methoxy group to the position C3 and a hydroxyl group to the position C4 of 4-phenyl-3,4-dihydroquinolin-2(1H)-one, might promote the osteogenic differentiation of BMSCs. To further investigate the effects of CHNQD-00603 on BMSCs, we performed a CCK-8 assay and qRT-PCR, alkaline phosphatase staining (ALP), and alizarin red S staining to assess the cytotoxicity and the ability of osteogenic differentiation of CHNQD-00603. The autophagy level was assessed and validated by WB, qRT-PCR, and transmission electron microscopy. Then, 3-methyladenine (3-MA) was added to further examine the role of autophagy. Based on the expression of autophagy-related genes, we predicted and examined the potential miRNAs by bioinformatics.

**Results:** CCK-8 assay showed that CHNQD-00603 at 1 μg/ml did not influence BMSCs activity. However, the proliferation rate decreased from the seventh day. qRT-PCR, ALP staining, ALP activity assay, and Alizarin red S staining showed that the best concentration of CHNQD-00603 to promote osteogenic differentiation was 1 μg/ml. Further investigations indicated that CHNQD-00603 activated autophagy, and the inhibition of autophagy by 3-MA attenuated CHNQD-00603-enhanced osteogenic differentiation. Subsequently, the findings from bioinformatics and qRT-PCR indicated

that miR-452-3p might be a regulator of autophagy and osteogenesis. Furthermore, we transfected BMSCs with miR-452-3p NC and mimics separately to further determine the function of miR-452-3p. The data showed that the overexpression of miR-452-3p moderated the level of autophagy and osteogenic differentiation of CHNQD-00603-treated BMSCs.

**Conclusion:** Our data suggested that CHNQD-00603 promoted the osteogenic differentiation of BMSCs by enhancing autophagy. Meanwhile, miR-452-3p played a regulatory role in this process.

**Keywords: osteogenesis, marine natural products, periodontitis, miRNAs, autophagy**

## INTRODUCTION

Periodontitis and peri-implantitis are frequent oral diseases and a significant cause of tooth loss and implant failure (Smeets et al., 2014; Cardoso et al., 2018). In addition, they severely affect the quality of patients' lives. At present, the treatment of periodontitis and peri-implantitis mainly focuses on prevention, basic treatment, and guided tissue regeneration (Jepsen et al., 2015; Graziani et al., 2017). BMSCs with low immune response are a key element of bone regeneration that can differentiate into osteoblasts, chondrocytes, and adipocytes in different microenvironments (Kokabu et al., 2016). Transplantation of stem cells by injecting cell suspensions or seeding cells onto scaffolds has been demonstrated to promote bone regeneration *in vivo* in animals (Sinder et al., 2020; Jiang et al., 2021). More importantly, studies have investigated BMSCs in periodontal tissue regeneration (Ledesma-Martínez et al., 2019). However, the challenge is that the limited ability of osteogenic differentiation and rapid aging limits their clinical application (de Witte et al., 2017). Recently, scholars have focused on the discovery of bioactive compounds that can induce osteogenic differentiation of BMSCs (Ringe et al., 2002). Marine natural products (MNPs) are an excellent resource due to their diversity and abundance.

Marine natural products (MNPs) from marine organisms provide abundant and promising resources for bone research and, have been reported to be closely associated with bone growth and healing (Carson and Clarke, 2018). In the early stage, our cooperative team isolated a series of 4-phenyl-3,4-dihydroquinolin-2(1H)-one alkaloids from a gorgonian-derived fungus, i.e., *Scopulariopsis* sp. (TA01-33) (Shao et al., 2015). In this study, we obtained various new derivatives by adding different functional groups to 4-phenyl-3,4-dihydroquinolin-2(1H)-one core. The structure of some derivatives is presented in **Figures 1A–H**. Some preliminary cytological experimental results from osteogenesis-related gene expression, alkaline phosphatase activity (ALP), and western blotting both showed that CHNQD-00603, one of the derivatives, could promote osteogenic differentiation of BMSCs (**Figures 1I–K**). Therefore, CHNQD-00603 might be an effective molecule in osteogenesis. However, its mechanism of action is still unclear. Therefore, the present study aimed to further determine the effect of CHNQD-00603 on osteogenic differentiation and the relevant mechanisms.

Autophagy plays an essential role in maintaining the energy and nutrient balance by recycling unnecessary or damaged organelles and proteins (Wong et al., 2020). In the past few years, significant progress has been made in understanding the mechanisms by which autophagy participates in life activities (Glick et al., 2010; Parzych and Klionsky, 2014). mTOR signaling pathway is believed to be associated with autophagy (Al-Bari and Xu, 2020). Interestingly, in the present study, the mTOR signaling pathway and PI3K-AKT signaling, which participate in autophagy regulation, were predicted to be associated with CHNQD-00603 (**Figure 3F**).

In this study, we evaluated the cytotoxicity of CHNQD-00603 and its ability to promote osteogenic differentiation of BMSCs. First, we predicted the possible target genes of CHNQD-00603 by SwissTargetPrediction according to the structure. The results are presented in **Table 1**. DAVID analysis indicated that the predicted gene of this derivative was connected to the signaling pathways of bone metabolism and autophagy, including signaling pathways regulating pluripotency of stem cells, osteoclast differentiation, VEGF signaling pathways, and mTOR signaling pathway (**Figure 3F**). Our data confirmed that CHNQD-00603 promoted the osteogenic differentiation of BMSCs. Next, we further found that the effect was regulated by the miR-452-3p-mediated autophagy pathway.

## MATERIALS AND METHODS

### Isolation and Culture of Bone Marrow Mesenchymal Stem Cells (BMSCs)

Two-month-old female Sprague-Dawley rats were used to isolate the BMSCs. The rats were anesthetized with 10% chloral hydrate and sacrificed. The femur and tibia were collected after disinfection with alcohol. Bone marrows from the femur and tibia of the rats were flushed out by α-minimum essential medium (α-MEM) under aseptic conditions. After centrifuging, BMSCs were transferred to culture flasks. The medium was changed every 3 days until the adherent cells grew to 80% of the culture flask. BMSCs at passage three were used to estimate the surface markers, including CD45, CD90, CD29, and CD11b/c (Elabscience, China) by flow cytometry. All the experimental protocols were approved by the Intramural Animal Use and Care Committee of the affiliated hospital of Qingdao University.

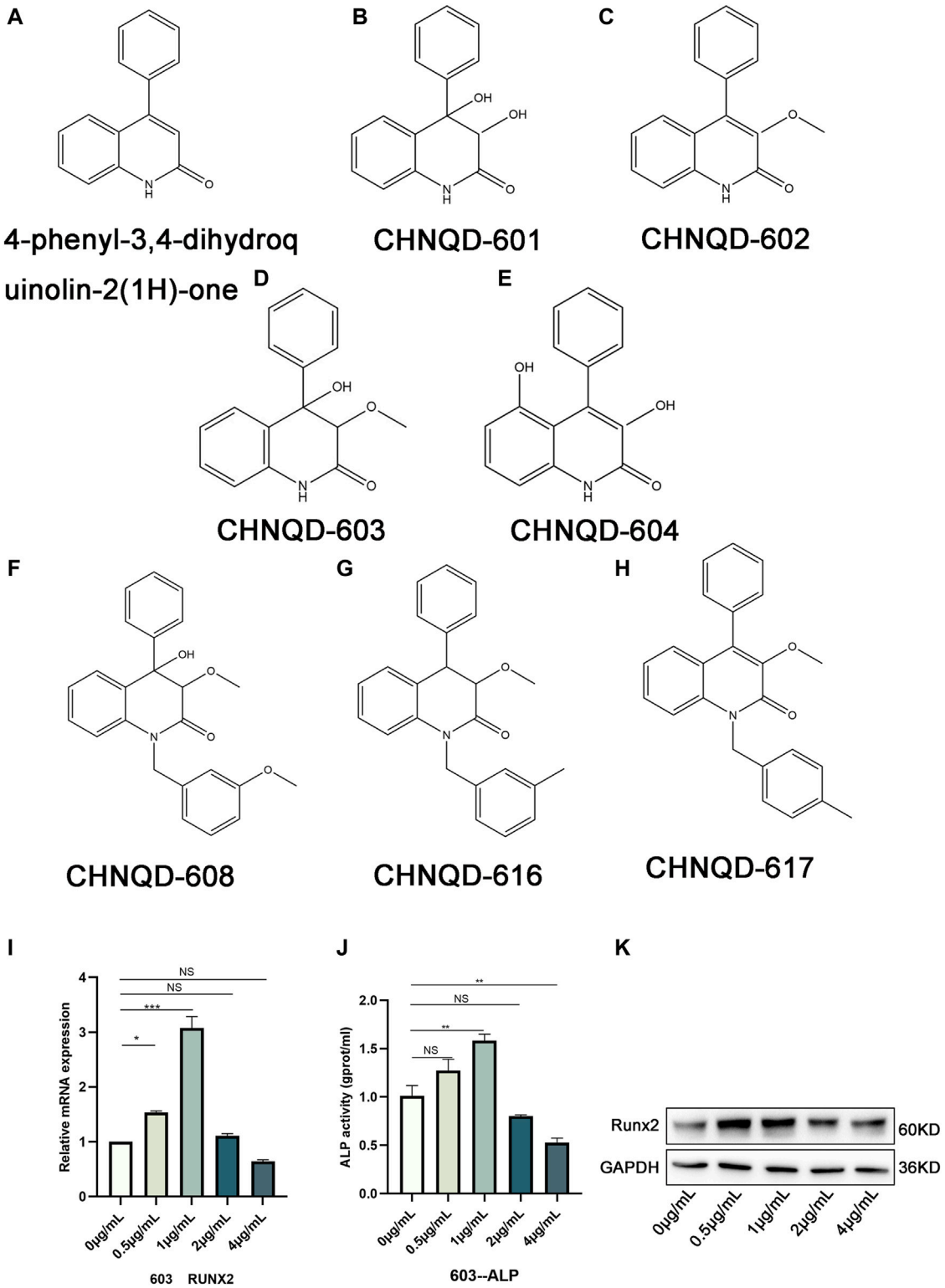


**FIGURE 1 |** The structure of 4-phenyl-3,4-dihydroquinolin-2(1H)-one and its derivatives. **(A)** The structure of 4-phenyl-3,4-dihydroquinolin-2(1H)-one. **(B–H)** Structures of a class of 4-phenyl-3,4-dihydroquinolin-2(1H)-one alkaloid derivatives. **(I)** qRT-PCR was used to observe the expression of the osteogenesis-related genes Runx2. **(J)** ALP activity assay was applied to access the early osteogenic differentiation of BMSCs. **(K)** WB was adopted to investigate the osteogenesis-related protein Runx2. Quantitative data were presented as the mean ± SD (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

**TABLE 1 |** Probable predictive target genes.

| – | – | – | – |
|---|---|---|---|
| CYP3A4 | EWS-Fli1 | EIF2AK3 | MAPK10 |
| CYP2C19 | ADRA1D | KDM5C | AHCY |
| CA2 | EDNRA | KDM4B | ALOX15 |
| CA1 | PSMB5 | KDM5B | SLC5A1 |
| TOP2A | BDKRB1 | KDM4A | PFKFB4 PFKFB3 |
| CES2 | JAK3 | CSNK2A1 | HMGCR |
| JAK1 | TYK2 | CA7 | FLT1 |
| JAK2 | PDE10A | CA6 | KIT |
| CASP1 | ABCC9 | CA12 | KDR |
| NFKBIA | LRRK2 | CA14 | MMP1 |
| RELA | EDNRB | CA9 | MMP2 |
| ERBB2 | FBP1 | CA4 | MMP8 |
| EGFR | ADRA2C | CA13 | DHODH |
| AOC3 | CDK5R1 CDK5 | CA5B | ADORA1 |
| CSF1R | ALOX12 | CA5A | PTAFR |
| ACPP | CYP51A1 | CDK2 CCNA1 CCNA2 | CDC7 |
| HSD17B2 | CAPN1 | CDK9 CCNT1 | – |

## Cell Counting Kit-8 (CCK-8) Analysis

The third-generation cells were seeded into a 96-well plate (7,000 cells/well) and cultured for 24 h. Then the medium was replaced with 100 μL of new medium added with different concentrations of CHNQD-00603 (0 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, and 4 μg/ml). The medium was changed every 3 days. After culturing for 3, 5, and 7 days, the cell proliferation and cell viability of BMSCs were examined by a CCK-8 detection kit following the manufacturer's instructions.

## Osteogenic Differentiation of BMSCs

The third-generation cells were placed in a six-well plate ($2\times10^5$ cells/well). The medium was replaced with osteogenic induction medium, consisting of α-MEM supplemented with 10% fetal bovine serum (HyClone, United States), 1% penicillin and streptomycin (Solarbio, China), dexamethasone (10 nM, Solarbio), beta-Glycerol phosphate (10 mM, Solarbio) and ascorbic acid 2-phosphate (200 μM, Solarbio) when the cell density reached 80% of the plate. The medium was changed every 3 days. ALP staining (Meilunbio, China) was performed 7 days later to observe the early osteogenic differentiation. Moreover, after 14 days, ARS (Solarbio, China) was used to assess late osteogenic differentiation according to the manufacturer's instructions. The staining was observed under a microscope at low magnification.

## Adipogenic and Chondrogenic Differentiation of BMSCs

The third-generation cells were placed in a six-well plate ($2\times10^5$ cells/well). The adipogenic differentiation medium (Procell, China) and chondrogenic differentiation medium (Procell, China) were added, respectively, according to the manufacturer's instructions after the cell density reached 80% of the plate. The medium was changed every 3 days. After 14 days, Oil Red O staining assay (VivaCell Bioscience) and Chondro-dye (VisaCell Biosciences) were used to observe the adipogenic differentiation and adipogenic differentiation under a microscope at low and high magnification.

## RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was obtained from the BMSCs using Trizol reagents. A PrimeScript RT (TaKaRa) reagent kit was applied to synthesize the cDNA. qRT-PCR was conducted by a CFX96 Real-Time System (BIO-RAD) using SYBR Premix Ex Taq (TaKaRa). The expression of mRNA was normalized to GAPDH or U6 using the $2^{-\Delta\Delta Ct}$ method. The primers are listed in **Table 2**.

## Western Blot

The specific methods have been introduced in detail in previous articles (Zhang et al., 2021). In brief, the cells were lysed with cell lysis buffer for Western and IP (Beyotime Biotechnology) supplemented with PMSF and cocktail on ice for 30 min. After centrifugation, the cell lysates were collected and analyzed with BCA Protein Assay Kit (Solarbio). Equal amounts of protein were added to SDS-containing polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich, China). 5% skimmed milk was utilized to block the membrane at room temperature for 2 h. Then the primary monoclonal antibodies were incubated with the membranes overnight at 4°C. Excessive antibodies were removed with TBST. Then, species-specific secondary antibodies were used to bind to the corresponding primary antibodies for 1 h at room temperature. The ChemiDoc Touch Imaging System (BioRad) was applied to collect images, and the ImageJ was used to quantify proteins.

## Alizarin Red S Staining, ALP Activity Assay, and ALP Staining

Alizarin Red solution (Solarbio, China) was applied to detect the effect of CHNQD-00603 on the osteogenic differentiation ability of BMSCs. The third-generation BMSCs were placed in a six-well plate ($2\times10^5$ cells/well). The different concentrations of CHNQD-00603 solutions were respectively added to cells after the cell density reached 80%. The medium was changed every 3 days. In addition, 3-MA (an inhibitor of autophagy), Rapa (an inhibitor of the mTOR pathway), and miR-452-3p were added in different experiments. After osteogenesis induction for 14 days, the cells were fixed by 4% paraformaldehyde and stained with Alizarin Red solution following the manufacturer's instructions. In addition, ALP activity assay and ALP staining were used to detect the early osteogenic differentiation. After culturing in the osteogenesis-inducing media for 7 days, the cells were detected and stained by ALP activity (Beyotime, China) and an alkaline phosphatase kit (Meilunbio, China).

## Prediction of Probable Target Genes and Signaling Pathway Analysis

Swiss TargetPrediction database (http://www.swisstargetprediction.ch/) was used to predict the possible target genes according to CHNQD-00603 structure. DAVID database (https://david.ncifcrf.gov/summary.jsp) was used to evaluate the probable signaling pathway by the predicted target genes.

**TABLE 2 |** The sequence of primers.

| Gene | Sequence |
|---|---|
| Runx2 | Forward:CTTCAAGGTTGTAGCCCTCG |
| | Reverse:TAGTTCT CATCATTCCCGGC |
| ALP | Forward:CTAGTTCCTGGGAGATGGTA |
| | Reverse:GTGTTGTACGTCTTGGAGAGA |
| OCN | Forward:CATGAGGACCCTCTCTCTGC |
| | Reverse:TGGACATGAAGGCTTTGTCA |
| ATG5 | Forward:TGGGATTGCAAAATGACAGA |
| | Reverse:TTCCCCATCTTCAGGATCAA |
| LC3 | Forward:TACCAAGGCAAAAAGGGACG |
| | Reverse:CCCCTGACACTGCTCTTCTAT |
| P62 | Forward:AGCTGCCCTCAGCCCTCT |
| | Reverse:GGCTTCTCTTCCCTCC |
| GAPDH | Forward:CCTCGTCTCATAGACAAGATGGT |
| | Reverse:GGGTAGAGTCATACTGGAACATG |
| ATG14 | Forward:TGCCGAACAATGGGGACTAC |
| | Reverse:AGGCAG GGTTGTTATGCTCC |
| Atg7 | Forward:TTTGTGGACAACGCCAAGATC |
| | Reverse:GAACCCGCTGGCATTCACT |
| Atg12 | Forward:ACCCGGACTGTCCAAGCA |
| | Reverse:ACCATCACTGCCAAAACACTCA |
| Atg16L1 | Forward:CATGGACCGCAGGGTTAAAC |
| | Reverse:CGGCTTGCAAAATCATTTGA |
| Beclin1 | Forward:CCAGACAGTGTTGTTGCTCCAT |
| | Reverse:CGCAAACCCCCAGAACAGTA |
| rno-miR-452-3P | Forward:GGCCTCAGTCTCATCTGCAAA |
| | RT:GTCGTATCCAGTGCAGGGTCCGAGG |
| | TATTCGCACTGGATACGACCTTCTT |
| rno-miR-6331 | Forward:GGGCTTTGGTGGCTTAGTTCTTT |
| | RT:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC |
| | GACGCACAA |
| U6 | Foward:CGCTTCGGCAGCACATATACTA |
| | RT:GGAACGCTTCACGAATTTGC |
| Universe R | CCAGTGCAGGGTCCGAGGT |

## Prediction of miRNA

miRDB database ((http://mirdb.org/) was applied to find the potential miRNA that could bind to the changed autophagy-related genes (Atg5 and Atg14). Cytoscape 3.6.1 was applied to draw the final visualization.

## Transmission Electron Microscopy

BMSCs ($2\times10^5$ cells/well) were treated with or without 1 μg/ml CHNQd-00603 for 7 days, or BMSCs ($2\times10^5$ cells/well) were transfected with miR-452-3p NC and mimics for 7 days. Then, the treated cells were fixed in 2.5% glutaraldehyde (Solarbio). The next steps were performed by Servicebio Company, and a transmission electron microscope was used to observe the results.

## Immunofluorescence Analysis

The pretreated cells were spread into small confocal dishes. After 48 h, the cells were fixed by 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 10 min. Then, 5% bovine serum albumin (FBS) was used to block the cells, and then LC3 (1:200, Proteintech) antibody was used to incubate the cells at 4°C overnight. The next day, the cells were incubated with the second antibody for 1 h, and the nucleus was stained with DAPI (Sigma-Aldrich) for 5 min. A laser scanning confocal microscope was used to observe the images.

## Cell Transfection

Third-passage BMSCs were transfected with negative control (NC) and miR-452-3p (mimics) by Lipofectamine 3,000 (Invitrogen, United States) according to the manufacturer's instructions. After transfecting for 48 h, transfection efficiency was determined by qRT-PCR.

## Statistical Analysis

All the corresponding experiments were independently repeated three times. The data were expressed in mean ± SD and analyzed by *t*-test and one-way ANOVA with Graphpad Prism 8.0. Bonferroni test was used as a post hoc test when the results analyzed by one-way ANOVA were significant. $p < 0.05$ was considered statistically significant.

# RESULTS

## The structures of 4-phenyl-3,4-dihydroquinolin-2(1H)-one alkaloid derivatives

**Figure 1** represents the structures of 4-phenyl-3,4-dihydroquinolin-2(1H)-one alkaloid and some of its derivatives. **Figure 1A** presents the structure of 4-phenyl-3,4-dihydroquinolin-2(1H)-one core. CHNQD-00601 was obtained by adding a hydroxyl group to the position C3 and position C4 of 4-phenyl-3,4-dihydroquinolin-2(1H)-one core (**Figure 1B**). CHNQD-00602 was obtained by adding a methoxy group to the position C3 of 4-phenyl-3,4-dihydroquinolin-2(1H)-one core (**Figure 1C**). CHNQD-00603 was gained by adding a methoxy group to the position C3 and a hydroxyl group to the position C4 of 4-phenyl-3,4-dihydroquinolin-2(1H)-one (**Figure 1D**). CHNQD-00604 was obtained by substituting hydrogen at positions C3 and C5 with hydroxyl groups (**Figure 1E**). The derivative CHNQD-00608 was formed by replacing the hydrogen of position N1, position C3, and position C4 with 1-(3-methoxy benzyl), methoxy, and hydroxyl groups, respectively (**Figure 1F**). The derivatives CHNQD-00616 and CHNQD-00617 both replaced hydrogen with a methoxy group at position C3, but the position N1 of CHNQD-00616 was 1-(3-methyl benzyl) while CHNQD-00617 was 1-(4-methyl benzyl) (**Figures 1G, H**). The results of some preliminary cytological experiments indicated that the expression of osteogenesis-related gene Runx2, ALP activity, and osteogenesis-related protein Runx2 had the same trend (**Figures 1I–K**). Therefore, we believe that CHNQD-00603 could promote osteogenic differentiation, and we chose CHNQD-00603 for further investigation.

## The Effects of CHNQD-00603 on Cell Viability and Osteogenic Differentiation of BMSCs

BMSCs isolated from the bone marrow were positive for CD29 and CD90 and negative for CD45 and CD11b/c (**Figure 2A**). The adipogenic differentiation and chondrogenic differentiation were

observed at low and high magnification, respectively. The results showed that fat cells were stained orange, and chondrocytes were stained blue (**Figures 2B, C**). ALP staining and ARS at low magnification indicated the formation of osteoblasts (**Figure 2D**). To estimate the response of BMSCs to CHNQD-00603, we treated the cells with different concentrations of CHNQD-00603 (0 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, and 4 μg/ml) and then detected the cell viability and cell proliferation with a CCK-8 reagent. After incubating for 5 and 7 days, CHNQD-00603 was shown to promote cell proliferation at a dose of 0.5 μg/ml compared with the untreated group. CHNQD-00603 did not affect cell proliferation at 1 μg/ml and 2 μg/ml after 3 and 5 days, but the cell proliferation rate began to decrease on day 7. However, CHNQD-00603 exhibited a noticeable inhibitory effect at a concentration of 4 μg/ml (**Figures 3A, B**). Subsequently, we examined the osteogenic differentiation of BMSCs treated with different doses of CHNQD-00603 after 7 days. Osteogenesis-related gene was determined by qRT-PCR. The results showed that the mRNA expression of alkaline phosphatase (ALP), osteocalcin (OCN), and Runt-related transcription factor 2 (RUNX2) increased at concentrations 0.5 μg/ml and 1 μg/ml, with 1 μg/ml showing the strongest effect on mRNA expression, which was two to three folds compared with the untreated group. Besides, there was no effect or inhibitory effect on mRNA expression at 2 and 4 μg/ml, respectively (**Figure 3C**). To further determine the effect of CHNQD-00603 on osteogenic differentiation, Alizarin red S staining (ARS) was analyzed. The results showed more calcium salt deposits at a concentration of 1 μg/ml than other concentrations on day 14 (**Figure 3D**). In addition, ALP activity, which indicated the early osteogenic differentiation of BMSCs, was assessed by staining and quantitative analysis after 7 days. Consistent with mRNA expression and ARS staining, there was a more significant ALP staining and a higher ALP quantitation at the concentration of 1 μg/ml (**Figure 3E**) and a lower increase at 0.5 μg/ml. Taken together, CHNQD-00603 exhibited no cytotoxicity to BMSCs but promoted osteogenic differentiation of BMSCs at a dose of 1 μg/ml. Therefore, we selected 1 μg/mL as a proper concentration for further experiments.

## CHNQD-00603 Activates Autophagy in the Osteogenic Differentiation of BMSCs

According to our prediction and analyses, autophagy-related signaling pathways-mTOR signaling pathway and PI3K-AKT signaling pathway might be associated with CHNQD-00603, which indicated that autophagy might be involved in the osteogenic differentiation of BMSCs treated with CHNQD-00603 (**Figure 3F**). Thus, we investigated the autophagy level in BMSCs treated with CHNQD-00603. Transmission electron microscopy (TEM) was used to observe autophagosomes. Compared with the untreated group, CHNQD-00603 significantly increased the number of autophagosomes in BMSCs on day 7 of osteogenesis (**Figure 4A**). QRT-PCR was then applied to detect the expression of autophagy-related genes (LC3 and p62). The results showed that the autophagy-related gene LC3 increased about 1.7-fold that in the untreated group. In addition, the expression of p62 was 0.3-fold that in the untreated group (**Figure 4B**). The autophagy-related protein LC3 was tested by western blot and quantitative assay and immunofluorescent assays to further estimate whether autophagy was elevated. We found that the expression of the autophagy-related protein LC3 increased in BMSCs treated with CHNQD-00603, and p62 was attenuated (**Figures 4C, D**). Accordingly, more LC3 dots were accumulated in BMSCs treated with CHNQD-00603 compared with the untreated group (**Figure 4E**). These data suggested that CHNQD-00603 activated autophagy.

## CHNQD-00603 Promotes Osteogenic Differentiation of BMSCs by Upregulating Autophagy

3-methyladenine (3-MA, an inhibitor of autophagy) was added to the control and CHNQD-00603 groups to change autophagic activity to examine the role of autophagy in CHNQD-00603-induced osteogenic differentiation of BMSCs. The results of western blotting and quantitative assay and qRT-PCR suggested that 5-μM 3-MA decreased the level of autophagy-related protein and mRNA of LC3 in the two groups, while the expression of p62 was enhanced (**Figures 5A–C**). Next, we further tested whether CHNQD-00603 promoted osteogenic differentiation by autophagy. QRT-PCR data showed that the expression of osteogenesis-related mRNAs (RUNX2, OCN, and ALP) was consistent with the level of autophagy. In particular, 3-MA attenuated CHNQD-00603-induced expression of osteogenesis-related genes (**Figure 5D**). Consequently, ALP staining and ALP activity indicated that the early osteogenic differentiation induced by CHNQD-00603 was inhibited by adding 3-MA (**Figure 5E**). ARS staining demonstrated that 3-MA decreased calcium salt deposits (**Figure 5F**). These findings revealed that autophagy plays an indispensable role in the CHNQD-00603-induced osteogenic differentiation.

Prediction and validation of miRNAs in CHNQD-00603-induced osteogenic differentiation of BMSCs.

We identified the autophagy-related genes (Atgs) by qRT-PCR to further estimate the expression of autophagy. The data showed that the expression of Atg5 and Atg14 was significantly higher than other genes, with three folds that of the untreated group (**Figure 6A**). Therefore, we presumed that there might be a factor regulating the expression of these two genes. Recently, interactions have been reported between miRNA and autophagy in bone homeostasis (Shen et al., 2016). Therefore, we supposed that CHNQD-00603 induces osteogenic differentiation of BMSCs by regulating miRNAs, which changed the level of autophagy by silencing the target gene. Hence, we selected Atg14 and Atg5 as target genes based on the expression level of the autophagy-related gene. Then, we searched the miRNAs that bind to Atg14 and Atg5 using the miRDB database. The results showed 51 miRNAs with binding sites for Atg14 and 26 miRNAs with binding sites for Atg5 (**Figure 6B**). Among these miRNAs, we found that miR-452-3p and miR-6331 could bind to Atg14 and Atg5 (**Figure 6B**). The complementary base pairing was shown in **Figure 6C**. Therefore, we hypothesized that miR-452-3p and miR-6331 regulate the expression of autophagy in this study. Subsequently, we

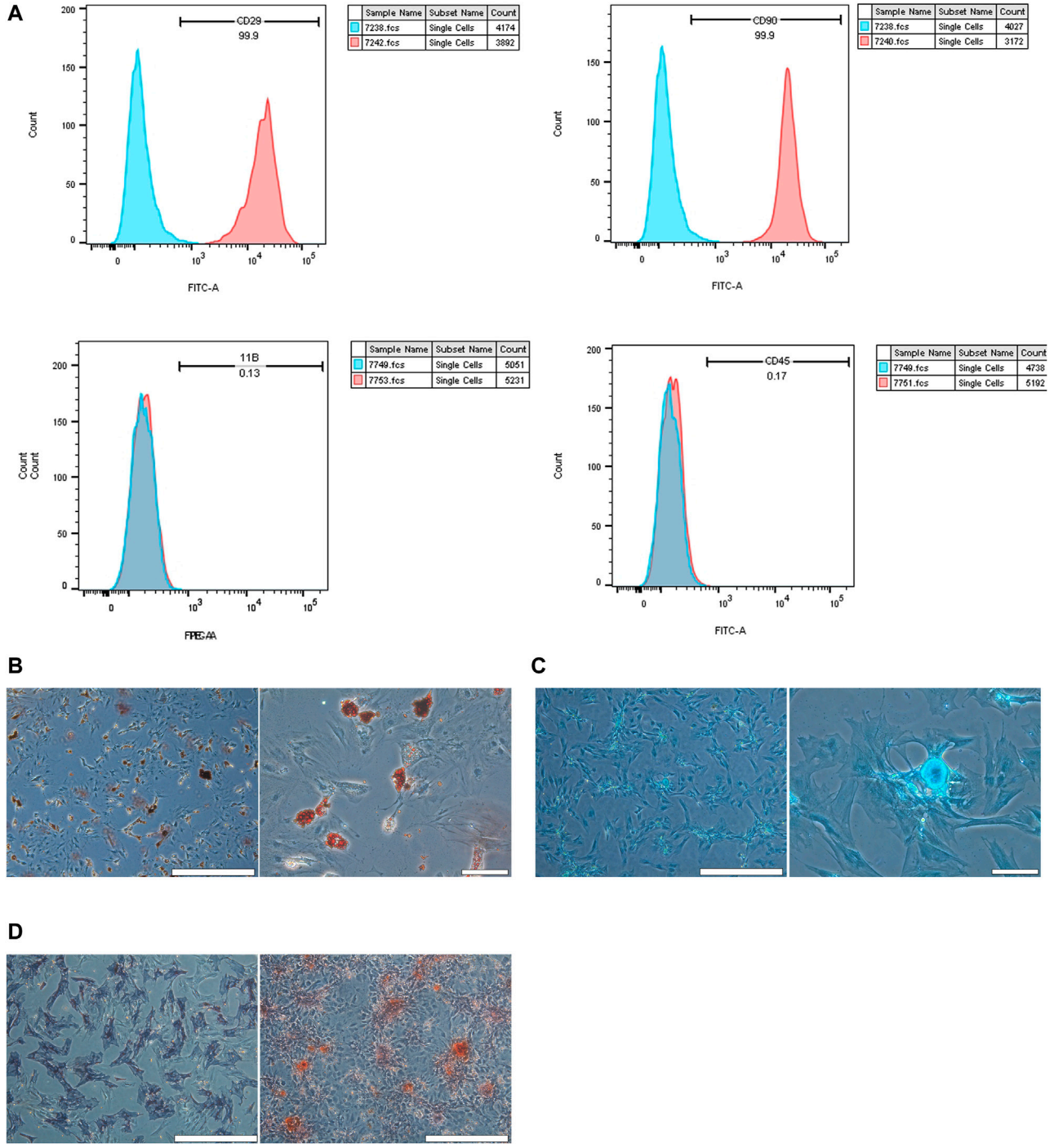


**FIGURE 2 |** Validation of BMSCs. **(A)** flow cytometry was used to confirm the characterization of BMSCs. BMSCs expressed CD90 and CD29, but they did not express CD45 and CD11b/c. **(B)** Adipogenic differentiation was identified by Oil Red O staining at low and high magnification. **(C)** Chondrogenic differentiation was assessed by chondroblast staining at low and high magnification. **(D)** Osteogenic differentiation was evaluated by ALP staining and ARS staining at low magnification (scale bar = 200 μm).

performed qRT-PCR to validate the expression of these two miRNAs. QRT-PCR assay indicated that miR-452-3p decreased to 0.5-fold in CHNQD-00603-induced osteogenic differentiation of BMSCs compared to the untread group. However, there was no modest change in the level of rno-miR-6331 (**Figure 6D**). These data suggested that miR-452-3p might participate in the regulation of CHNQD-00603-induced osteogenic differentiation of BMSCs.

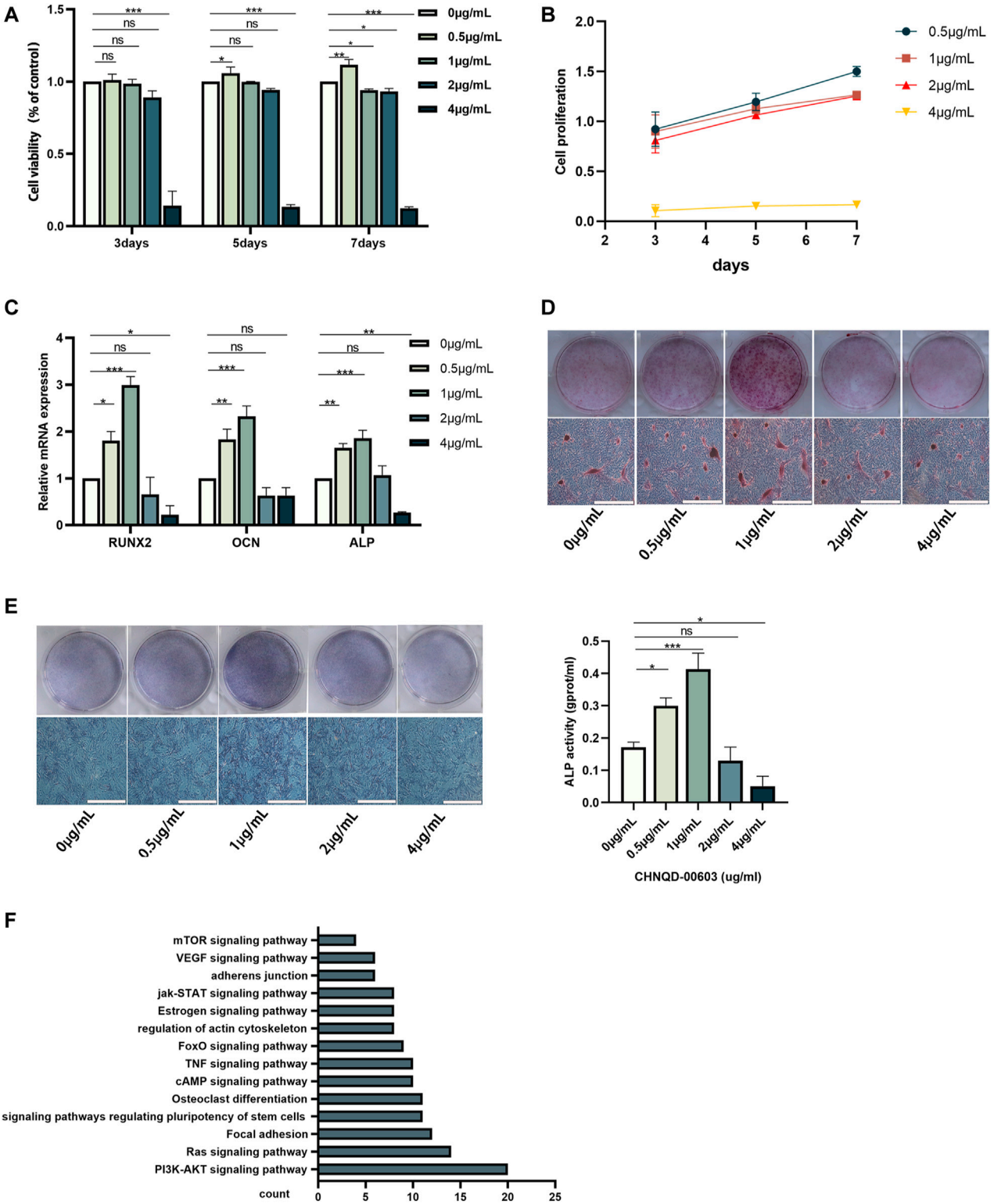


**FIGURE 3 |** The effect of different concentrations of CHNQD-00603 on cell viability and osteogenic differentiation of BMSCs. **(A, B)** CCK-8 assay was performed to detect cell viability and cell proliferation of BMSCs cultured with different concentrations of CHNQD-00603 (0 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, and 4 μg/ml) for 3, 5, and 7 days **(C)** qRT-PCR tested the expression of osteogenesis-related genes in CHNQD-00603-induced BMSCs on day 7. **(D)** Alizarin red S staining indicated calcium salt deposits on day 14. **(E)** ALP staining and ALP activity were evaluated on day 7 (scale bar = 200 μm, scar bar = 200 μm). **(F)** Bioinformatic analysis of predicted target genes. Quantitative data are presented as the mean ± SD (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

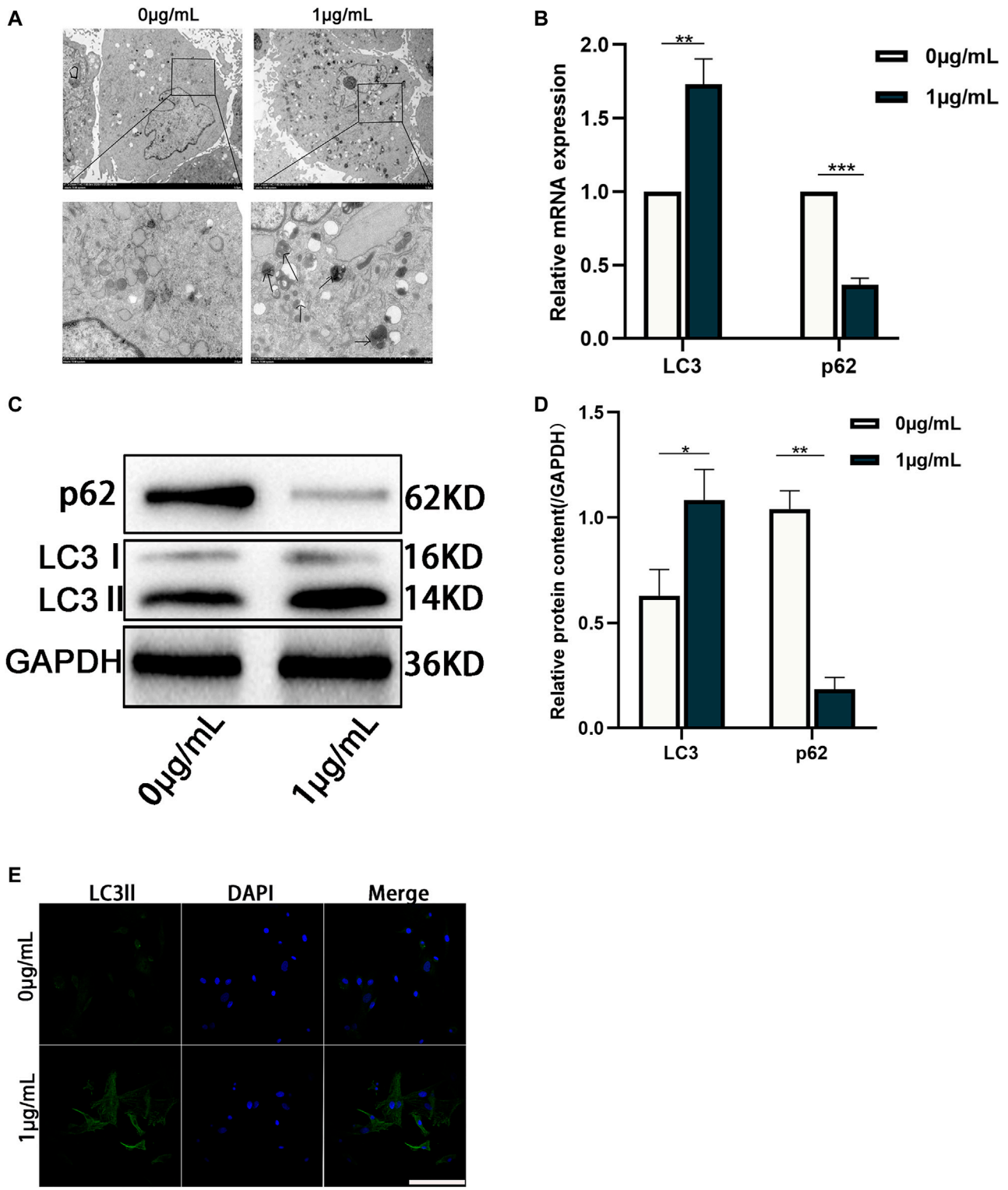


**FIGURE 4 |** Autophagy is activated in the osteogenic differentiation of BMSCs treated with CHNQD-00603. **(A)** TEM was used to observe the number of autophagosomes on day 7. **(B)** qRT-PCR was performed to detect the expression of autophagy-related gene LC3 and p62. **(C,D)** Western blot and quantitation were performed to analyze the expression of proteins LC3 and p62. **(E)** Immunofluorescence staining was used to observe the distribution of LC3 (scale bar = 50 μm). Quantitative data are presented as the mean ± SD (n = 3) (**$p < 0.01$, ***$p < 0.0001$).

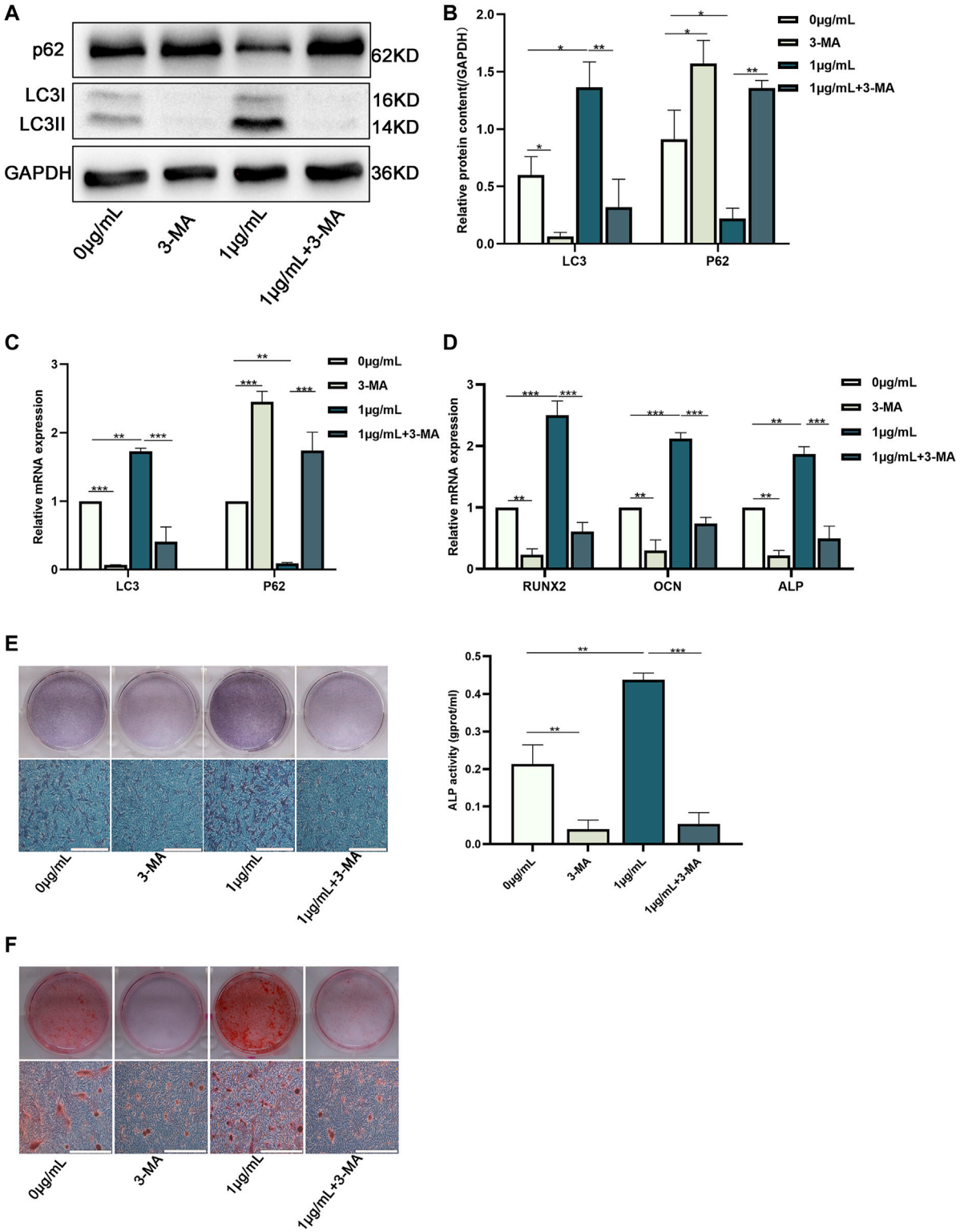


**FIGURE 5 |** Downregulation of autophagy inhibited the osteogenic differentiation of BMSCs treated with CHNQD-00603. BMSCs cultured with or without CHNQD-00603 were treated with 5-μM 3-MA and then induced for osteogenic differentiation. The level of autophagy and osteogenesis was analyzed on day 7. **(A,B)** Autophagy-related proteins LC3 and p62 were detected by Western blot and quantitation. **(C)** Autophagy-related genes LC3 and p62 were assessed by qRT-PCR. **(D)** Osteogenesis-related genes Runx2, OCN, and ALP were tested by qRT-PCR. **(E)** ALP staining and ALP activity assay indicated early osteogenic differentiation (scale bar = 200 μm). **(F)** ARS was used to detect calcium salt deposits on day 14 (scale bar = 200 μm). Quantitative data are presented as the mean ± SD (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

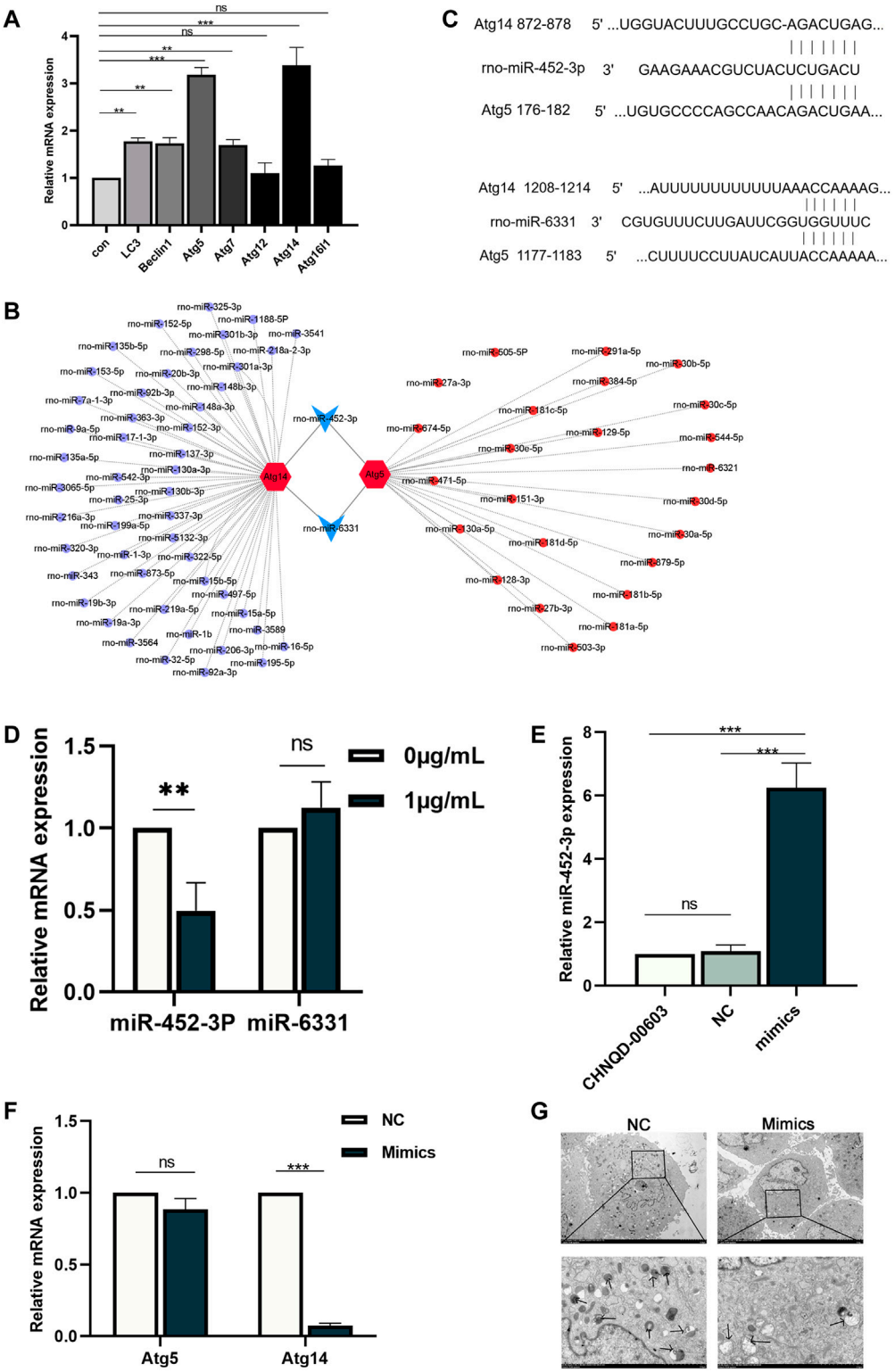


**FIGURE 6 |** Prediction and validation of potential miRNAs and overexpression of miR-452-3p. **(A)** Autophagy-related genes were estimated by qRT-PCR. **(B)** The targeting network was constructed using Cytoscape. **(C)** The complementary base pairing of miR-452-3p and miR-6331 with Atg5 and Atg14, respectively. **(D)** qRT-PCR was performed to detect the expression of miR-452-3p and miR-6331 in the osteogenesis of BMSCs treated with CHNQD-00603. Upregulation of miR-452-3p attenuates the level of autophagy. **(E)** The efficiency of transfecting miR-452-3p mimics was evaluated by qRT-PCR 48 h after transfecting. **(F)** Autophagy-related genes Atg5 and Atg14 were detected after transfecting miR-452-3p mimics by qRT-PCR on day 7. **(G)** The number of autophagosomes on day 7 was observed by TEM. Quantitative data are presented as the mean ± SD (n = 3) (**$p < 0.01$, ***$p < 0.0001$).

## Overexpression of miR-452-3p Decreases the Level of Autophagy

We transfected CHNQD-00603-treated BMSCs with miR-452-3p mimics and NC to explore the effect of miR-452-3p on autophagy. After 48 h, the qRT-PCR data showed that the expression of miR-452-3p had increased six folds in BMSCs transduced by miR-452-3p mimics compared with the NC and controls (**Figure 6E**). Then we further detected the level of autophagy by qRT-PCR and transmission electron microscopy (TEM). The results revealed that miR-452-3p mimics decreased the expression of Atg14 to 0.1-fold compared with NC, but the downregulation of Atg5 was not different (**Figure 6F**). Accordingly, the number of autophagosomes decreased significantly (**Figure 6G**). Thus, our findings indicated that miR-452-3p regulated autophagy levels in BMSCs treated with CHNQD-00603.

## CHNQD-00603 Promotes Osteogenic Differentiation by miR-452-3p Medicating Autophagy

We used miR-452-3p mimics and Rapamycin in BMSCs cultured with CHNQD-00603 to verify whether osteogenic differentiation induced by CHNQD-00603 was mediated by miR-452-3p regulating autophagy. The results of qRT-PCR and Western blot revealed that transfection of miR-452-3p mimics attenuated the expression of osteogenesis-related mRNA (Runx2, ALP, and OCN) and protein (Runx2 and ALP). In addition, the increased expression of these genes and proteins by Rapa was inhibited by miR-452-3p mimics (**Figures 7A–C**). ALP activity and ARS consistently showed the same tendency as qRT-PCR and western blotting (**Figures 7D, E**). Thus, our findings indicated that miR-452-3p targeted autophagy to regulate CHNQD-00603-induced osteogenic differentiation of BMSCs.

# DISCUSSION

Marine natural products are abundant concerning source and variety. A class of derivatives can be obtained by structural optimization with different biological activities, including anti-tumor, anti-inflammatory, and bone metabolism effects. In this study, we obtained several new derivatives by adding different groups to different positions of 4-phenyl-3,4-dihydroquinolin-2(1H)-one core. Preliminary cytological experiments indicated that CHNQD-00603 could potentially promote osteogenic differentiation of BMSCs, as evidenced by the increase in osteogenesis-related gene and proteins Runx2 and early osteogenic differentiation marker ALP activity. By bioinformatics, we found a relationship between CHNQD-00603 and bone metabolism. Therefore, we chose CHNQD-00603 for further investigation. The results of cytotoxicity and osteogenic differentiation experiments showed a lower rate of proliferation of BMSCs on day 7 at a concentration of 1 μg/ml. However, the osteogenic differentiation was strongest, evidenced by the results of qRT-PCR, ARS and ALP staining, and ALP activity assay. Therefore, we believe that the decrease in the cell proliferation rate was associated with early osteogenic differentiation. Furthermore, it is believed that dehydrogenase activity was always relatively low during cell differentiation (Hao et al., 2019). These data demonstrated the role of CHNQD-00603 in osteogenesis for the first time. Thus, the present study provided a groundbreaking choice for bone regeneration.

Autophagy, a fundamental process, sequesters intracellular constituents to deliver them to lysosomes that degrade the intracellular components and recycle them into macromolecule precursors (Chang, 2020). Defective autophagy leads to the senescence of mesenchymal stem cells and bone loss (Li et al., 2018; Yang et al., 2020). Thus, autophagy is an indispensable process in osteogenic differentiation. Autophagy-related signaling molecules, mTOR and PI3K-Akt, were hypothesized to be associated with CHNQD-00603 (**Figure 3F**). Therefore, we investigated whether autophagy participated in the osteogenic differentiation of CHNQD-00603-treated BMSCs. Our data showed that autophagy was activated in CHNQD-00603-treated BMSCs. In addition, the inhibition of autophagy by 3-MA attenuated osteogenic differentiation. Together with our prediction results, these data indicated that autophagy plays a crucial role in the osteogenic differentiation of BMSCs induced by CHNQD-00603. Our study proposed for the first time that derivatives from 4-phenyl-3,4-dihydroquinolin-2(1H)-one could activate autophagy, promoting the osteogenic differentiation of BMSCs. This provided a new direction to study bone regeneration.

Autophagy-related genes, a group of evolutionarily conserved genes, are involved in the formation and regulation of the autophagy process by communicating with intracellular signaling pathways (Lan et al., 2021). We investigated the expression of a series of autophagy-related genes by qRT-PCR. The results revealed that the expression of Atg5 and Atg14 increased significantly in CHNQD-00603-treated BMSCs. Therefore, we believe that CHNQD-00603 activated autophagy mainly by promoting the expression of Atg5 and Atg14. It has been reported that miRNAs are important regulators of autophagy (Huang et al., 2020; Silwal et al., 2020). Moreover, miRNA is involved in osteogenic differentiation by altering autophagy (Liu et al., 2021). However, the effect of miRNAs on CHNQD-00603-induced autophagy and osteogenic differentiation is still mysterious. Here, by searching the miRDB database and visualizing with a Cytoscape, we found two miRNAs with a targeted contact relationship with both Atg14 and Atg5, including miR-452-3p and miR-6331. Further investigation by qRT-PCR showed that the expression of miR-452-3p decreased in CHNQD-00603-treated BMSCs, but the expression of miR-6331 was not different in CHNQD-00603-treated BMSCs compared with non-treated BMSCs. Therefore, we selected miR-452-3P to further explore its role in CHNQD-00603-induced autophagy and osteogenic differentiation.

We overexpressed miR-452-3p by mimics in BMSCs induced by CHNQD-00603 to determine whether miR-452-3p could regulate autophagy in CHNQD-00603-treated BMSCs. We found that the autophagy activity was suppressed, as

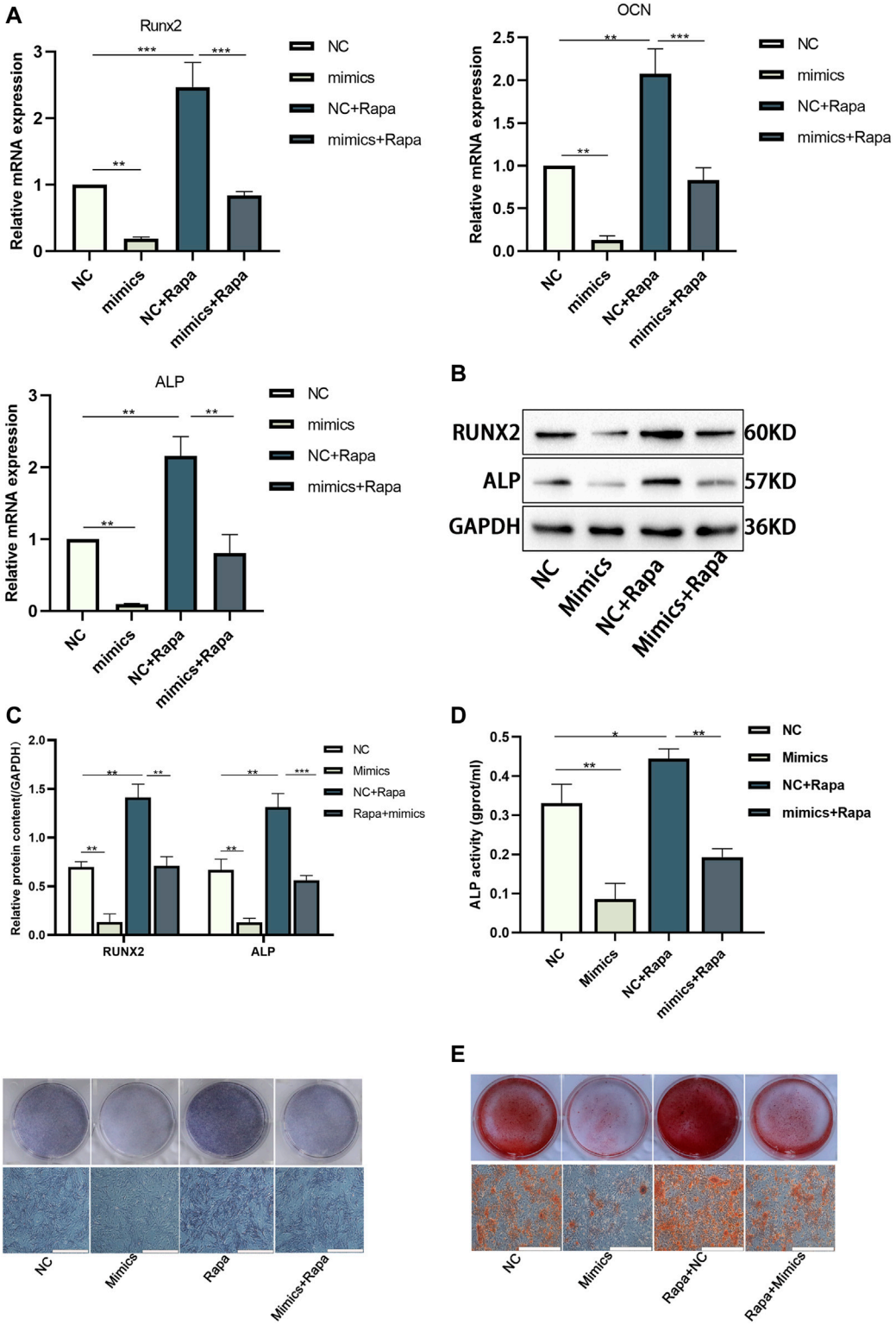


**FIGURE 7 |** The osteogenic differentiation elevated by Rapa was further disturbed by transfecting miR-452-3p mimics in BMSCs treated with CHNQD-00603. BMSCs treated with CHNQD-00603 were divided into four subgroups: NC, mimics, NC + Rapa, and mimics + Rapa. **(A–C)** Osteogenesis-related genes and proteins were detected by qRT-PCR and Western blot and protein quantification on day 7. **(D)** ALP activity assay and ALP staining were applied to evaluate the early osteogenic differentiation (scale bar = 200 μm). **(E)** ARS was used to estimate calcium salt deposits (scale bar = 200 μm). Quantitative data are presented as the mean ± SD (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

evidenced by a decreased number of autophagosomes and autophagy-related genes. In particular, the expression of Atg14 was visibly weakened. We changed autophagy activity by mTOR inhibitor (rapamycin) to identify the relationship between osteogenic differentiation and miR-452-3p-regulated autophagy. The results showed that upregulated osteogenic differentiation by Rapa was inhibited by miR-452-3p mimics. We found and identified a new miRNA, miR-452-3p, which inhibited autophagy. CHNQD-00603 promoted osteogenic differentiation by activating autophagy. However, the expression of miR-452-3p was inversely related to the level of autophagy and CHNQD-00603. Therefore, we believe that CHNQD-00603 plays a vital role in the osteogenic differentiation of BMSCs by miR-452-mediated autophagy.

## CONCLUSION

In this study, we demonstrated for the first time that CHNQD-00603 could promote the osteogenic differentiation of BMSCs. Furthermore, our research showed for the first time that CHNQD-00603 promoted osteogenic differentiation by miR-452-3p-regulated autophagy. Thus, the present study provided a theoretical basis for the clinical application of marine natural products.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Intramural Animal Use and Care Committee of the affiliated hospital of Qingdao university.

## AUTHOR CONTRIBUTIONS

All authors contributed to the study's conception and design. Material preparation and data collection were performed by SX, LG, and S-ML. Derivatives were synthesized and optimized by C-LS and Y-WW. The data analysis and manuscript revision were performed by J-JZ and W-HR. The first draft of the manuscript was written by SX. All authors gave their final approval and agreed to be accountable for all aspects of the work.

## FUNDING

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## REFERENCES

Al-Bari, M. A. A., and Xu, P. (2020). Molecular Regulation of Autophagy Machinery by mTOR-dependent and -independent Pathways. *Ann. N. Y Acad. Sci.* 1467, 3–20. doi:10.1111/nyas.14305

Cardoso, E. M., Reis, C., and Manzanares-Céspedes, M. C. (2018). Chronic Periodontitis, Inflammatory Cytokines, and Interrelationship with Other Chronic Diseases. *Postgrad. Med.* 130, 98–104. doi:10.1080/00325481.2018.1396876

Carson, M. A., and Clarke, S. A. (2018). Bioactive Compounds from Marine Organisms: Potential for Bone Growth and Healing. *Mar. Drugs* 16, 340. doi:10.3390/md16090340

Chang, N. C. (2020). Autophagy and Stem Cells: Self-Eating for Self-Renewal. *Front. Cell Dev. Biol.* 8, 138. doi:10.3389/fcell.2020.00138

de Witte, S. F. H., Lambert, E. E., Merino, A., Strini, T., Douben, H. J. C. W., O'Flynn, L., et al. (2017). Aging of Bone Marrow- and Umbilical Cord-Derived Mesenchymal Stromal Cells during Expansion. *Cytotherapy* 19, 798–807. doi:10.1016/j.jcyt.2017.03.071

Glick, D., Barth, S., and Macleod, K. F. (2010). Autophagy: Cellular and Molecular Mechanisms. *J. Pathol.* 221, 3–12. doi:10.1002/path.2697

Graziani, F., Karapetsa, D., Alonso, B., and Herrera, D. (2017). Nonsurgical and Surgical Treatment of Periodontitis: How many Options for One Disease? *Periodontol. 2000* 75, 152–188. doi:10.1111/prd.12201

Hao, M., He, J., Wang, C., Wang, C., Ma, B., Zhang, S., et al. (2019). Effect of Hydroxyapatite Nanorods on the Fate of Human Adipose-Derived Stem Cells Assessed *In Situ* at the Single Cell Level with a High-Throughput, Real-Time Microfluidic Chip. *Small* 15, e1905001. doi:10.1002/smll.201905001

Huang, T., Xu, Z., Cao, L., Lian, Y., and Huang, Y. (2020). Autophagy Regulation by microRNAs in Chemotherapy Resistance (Review). *Oncol. Rep.* 44, 791–797. doi:10.3892/or.2020.7655

Jepsen, S., Berglundh, T., Genco, R., Aass, A. M., Demirel, K., Derks, J., et al. (2015). Primary Prevention of Peri-Implantitis: Managing Peri-Implant Mucositis. *J. Clin. Periodontol.* 42 (Suppl. 16), S152–S157. doi:10.1111/jcpe.12369

Jiang, Y., Zhang, P., Zhang, X., Lv, L., and Zhou, Y. (2021). Advances in Mesenchymal Stem Cell Transplantation for the Treatment of Osteoporosis. *Cell Prolif* 54, e12956. doi:10.1111/cpr.12956

Kokabu, S., Lowery, J. W., and Jimi, E. (2016). Cell Fate and Differentiation of Bone Marrow Mesenchymal Stem Cells. *Stem Cells Int* 2016, 3753581. doi:10.1155/2016/3753581

Lan, T., Shiyu-Hu, H., Shen, Z., Yan, B., and Chen, J. (2021). New Insights into the Interplay between miRNAs and Autophagy in the Aging of Intervertebral Discs. *Ageing Res. Rev.* 65, 101227. doi:10.1016/j.arr.2020.101227

Ledesma-Martínez, E., Mendoza-Núñez, V. M., and Santiago-Osorio, E. (2019). Mesenchymal Stem Cells for Periodontal Tissue Regeneration in Elderly Patients. *J. Gerontol. A. Biol. Sci. Med. Sci.* 74, 1351–1358. doi:10.1093/gerona/gly227

Li, H., Li, D., Ma, Z., Qian, Z., Kang, X., Jin, X., et al. (2018). Defective Autophagy in Osteoblasts Induces Endoplasmic Reticulum Stress and Causes Remarkable Bone Loss. *Autophagy* 14, 1726–1741. doi:10.1080/15548627.2018.1483807

Liu, D., Wang, B., Qiu, M., and Huang, Y. (2021). MiR-19b-3p Accelerates Bone Loss after Spinal Cord Injury by Suppressing Osteogenesis via Regulating PTEN/Akt/mTOR Signalling. *J. Cell Mol Med* 25, 990–1000. doi:10.1111/jcmm.16159

Parzych, K. R., and Klionsky, D. J. (2014). An Overview of Autophagy: Morphology, Mechanism, and Regulation. *Antioxid. Redox Signaling* 20, 460–473. doi:10.1089/ars.2013.5371

Ringe, J., Kaps, C., Burmester, G.-R. x. d., and Sittinger, M. (2002). Stem Cells for Regenerative Medicine: Advances in the Engineering of Tissues and Organs. *Naturwissenschaften* 89, 338–351. doi:10.1007/s00114-002-0344-9

Shao, C.-L., Xu, R.-F., Wang, C.-Y., Qian, P.-Y., Wang, K.-L., and Wei, M.-Y. (2015). Potent Antifouling Marine Dihydroquinolin-2(1h)-One-Containing Alkaloids from the Gorgonian Coral-Derived Fungus Scopulariopsis Sp. *Mar. Biotechnol.* 17, 408–415. doi:10.1007/s10126-015-9628-x

Shen, G., Ren, H., Qiu, T., Liang, D., Xie, B., Zhang, Z., et al. (2016). Implications of the Interaction between miRNAs and Autophagy in Osteoporosis. *Calcif Tissue Int.* 99, 1–12. doi:10.1007/s00223-016-0122-x

Silwal, P., Kim, Y. S., Basu, J., and Jo, E.-K. (2020). The Roles of microRNAs in Regulation of Autophagy during Bacterial Infection. *Semin. Cell Developmental Biol.* 101, 51–58. doi:10.1016/j.semcdb.2019.07.011

Sinder, B. P., Novak, S., Wee, N. K. Y., Basile, M., Maye, P., Matthews, B. G., et al. (2020). Engraftment of Skeletal Progenitor Cells by Bone-Directed Transplantation Improves Osteogenesis Imperfecta Murine Bone Phenotype. *Stem Cells* 38, 530–541. doi:10.1002/stem.3133

Smeets, R., Henningsen, A., Jung, O., Heiland, M., Hammächer, C., and Stein, J. M. (2014). Definition, Etiology, Prevention and Treatment of Peri-Implantitis - a Review. *Head Face Med.* 10, 34. doi:10.1186/1746-160x-10-34

Wong, S. Q., Kumar, A. V., Mills, J., and Lapierre, L. R. (2020). Autophagy in Aging and Longevity. *Hum. Genet.* 139, 277–290. doi:10.1007/s00439-019-02031-7

Yang, Y., Lin, Z., Cheng, J., Ding, S., Mao, W. w., Shi, S., et al. (2020). The Roles of Autophagy in Osteogenic Differentiation in Rat Ligamentum Fibroblasts: Evidence and Possible Implications. *FASEB j.* 34, 8876–8886. doi:10.1096/fj.201903216rr

Zhang, W., Guo, J., Gao, L., Ren, W., Li, S-M., Zheng, J., et al. (2021). Upregulation of Mmu_circ_0001066 Attenuates the Inhibitory Effect of Bisphosphonates on Osteoclastogenesis. *Oral Dis*. Online ahead of print. doi:10.1111/odi.13871

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REVIEW
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# Recent Advances in Single-Cell View of Mesenchymal Stem Cell in Osteogenesis

*Fangyuan Shen and Yu Shi**

*State Key Laboratory of Oral Diseases and National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, China*

Osteoblasts continuously replenished by osteoblast progenitor cells form the basis of bone development, maintenance, and regeneration. Mesenchymal stem cells (MSCs) from various tissues can differentiate into the progenitor cell of osteogenic lineage and serve as the main source of osteoblasts. They also respond flexibly to regenerative and anabolic signals emitted by the surrounding microenvironment, thereby maintaining bone homeostasis and participating in bone remodeling. However, MSCs exhibit heterogeneity at multiple levels including different tissue sources and subpopulations which exhibit diversified gene expression and differentiation capacity, and surface markers used to predict cell differentiation potential remain to be further elucidated. The rapid advancement of lineage tracing methods and single-cell technology has made substantial progress in the characterization of osteogenic stem/progenitor cell populations in MSCs. Here, we reviewed the research progress of scRNA-seq technology in the identification of osteogenic markers and differentiation pathways, MSC-related new insights drawn from single-cell technology combined with experimental technology, and recent findings regarding the interaction between stem cell fate and niche in homeostasis and pathological process.

**Keywords: mesenchymal stem cells, osteogenesis, lineage tracing, single-cell, niche**

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*University of Copenhagen, Denmark*
*Ye Li,*
*University of Michigan, United States*

***Correspondence:**
*Yu Shi*
*yushi1105@scu.edu.cn*

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## 1 INTRODUCTION

The bone formation depends on the activation and recruitment of osteogenic stem/progenitor cells during bone development, reconstruction, and fracture repair. During embryogenesis, mesoderm-derived limb bud mesenchymal progenitors (LMPs) differentiate into osteochondrogenic lineages and generate primitive cartilage templates. After LMPs differentiate into cartilage, long bones are built through endochondral ossification. At the beginning of primary ossification center formation, perichondrial progenitor cells and blood vessels extend into cartilage lacuna left by the degeneration

**Abbreviations:** LMPs, Limb Bud Mesenchymal Progenitors; POC, Primary Ossification Center; SSCs, Skeletal Stem Cells; OCPs, Osteo-chondrogenic Progenitors; MSCs, Mesenchymal Stem Cells; mSSCs, Mouse Skeletal Stem Cell; WPC, Weeks Post Conception; eSSPCs, Embryonic Skeletal Stem/Progenitor Cells; NCDCs, Neural Crest-derived Cells; PDLSCs, Periodontal Ligament Stem Cells; P-SSCs, Periosteal Skeletal Stem Cells; BMSCs, Bone Marrow Mesenchymal Cells; PSCs, periosteal Stem Cell; OLCs, Osteolineage Cells; mpMSCs, Mesenchymal Stem Cells From The Metaphysis; dpMSCs, Mesenchymal Stem Cells From The Diaphysis; SuSCs, Suture Stem Cells; MMPs, Metaphyseal Mesenchymal Progenitor Cells; BMP, Bone Morphogenetic Protein; CTSK, Cathepsin K; Sca1, Stem Cell Antigen 1; CXCL12, C-X-C Motif Chemokine Ligand 12; EMPs, Early Mesenchymal Progenitors; TME, Tumors Microenvironment; ECM, Extracellular Matrix; SSPCs, Skeletal Stem/progenitor Cells; HO, Heterotopic Ossification.

of chondrocytes in the cartilage template, and then new bone marrow is formed (Maes et al., 2010; Ono et al., 2014). In postnatal life, bone is a dynamic tissue that is constantly being resorbed and remodeled. The undifferentiated MSCs in the bone marrow stroma are ancestors of osteoprogenitor cells and pre-osteoblasts, and under the regulation of Runt-related transcription factor 2 (*Runx2*) and Osterix (*Osx*) (Nakashima et al., 2002; Lian et al., 2006), they differentiate into mature osteoblasts which have a limited lifespan and are constantly replenished by osteogenic precursor cells.

MSCs have long been regarded as a direct source of osteogenic lineage progenitor cells in bone tissue. Their source is complex and correlates with stage and tissue specificity. MSC can be isolated from tissues such as teeth, bone marrow, adipose, umbilical cord, etc. The distribution, differentiation direction, immunosuppressive ability of MSCs subgroups from different sources are various, and the expression pattern also changes with age. Extensive studies have shown that MSCs are heterogeneous mixtures of multiple stem cell lineages, which can differentiate into osteoblasts, chondrocytes, and bone marrow stromal cells, fat cells, muscle cells, and endothelial cells (Uccelli et al., 2008; Chen et al., 2019). To identify cell subsets with specific functions in heterogeneous MSCs, cell surface markers are continuously explored. However, the non-negligible heterogeneities and lack of stage-specific markers hindered the identification and positioning of cell types and formed a significant barrier to our understanding of MSC populations. Until 2018, single-cell and lineage tracing technology was used to identify self-renewing and multipotent skeletal stem cells (SSCs) which could only differentiate into the progenitor of the osteogenic lineage (osteoprogenitors, chondroprogenitors, and progenitor cells of stroma) (Chan et al., 2018). With the emergence of numerous MSC-related studies, researchers have meticulously named the subgroups of MSCs such as embryonic Skeletal Stem/progenitor Cell (eSSPC), Bone Marrow Mesenchymal Cell (BMSC), Periosteal Stem Cell (PSC), etc., based on tissue origin, the developmental stage of donors, and differentiation characteristics. Possessing functional properties consistent with MSC, these renamed subgroups belong to the category of MSC.

Due to the rapid progress of mouse lineage-tracking techniques and single-cell RNA sequencing (scRNA-seq), researchers have made extraordinary progress in identifying and characterizing MSC heterogeneity and reconstructing osteogenic regulatory networks. To date, MSCs have been detected in growth plate cartilage, bone marrow stroma, the superficial layer of meniscus, bone surface resting-state bone lining cells and specialized fibroblasts in the craniofacial structure and have been characterized by several markers (i.e., leptin receptor [*LepR*], cathepsin K [*Ctsk*], glioma-associated oncogene homolog 1 [*Gli1*], and platelet-derived growth factor receptor [*Pdgfr*], etc.) *in vivo* (Maruyama et al., 2016; Yue et al., 2016; Shi et al., 2017; Mizuhashi et al., 2018; Baryawno et al., 2019; Bohm et al., 2019; Ortinau et al., 2019; Ponte et al., 2020; Wei et al., 2021; Zhang et al., 2021). In this review, we summarized the heterogeneity of MSCs derived from limb buds and postnatal distinct hard tissues as determined by single-cell resolution studies and the biologic functions and characteristics of new labeled stem/progenitor subpopulations. We also focused on the research progress on the osteogenic subpopulation of cre-targeted MSCs and the interaction between the osteogenic niche and precursor cells.

## 2 TRADITIONAL MSCS IDENTIFICATION METHODS

Current knowledge of MSCs mainly originates from experiments with human and rodent bone marrow cells. *In vitro* cultivation and allogeneic cell transplantation have become the gold standard for identifying MSC. Early studies verified the adherence, the ability to form fibroblast colonies (CFU-F), and the osteogenic ability after allograft transplantation to identify the proliferation and trilineage differentiation potential of MSCs (Cao et al., 2020). Flow cytometry was used to screen cell surface markers (L. Ramos et al., 2016). In recent years, many studies have begun to use lineage-tracing techniques to track the activity of MSC maker+ cells in transgenic mice, thereby identifying a series of MSC markers, such as *Grem1* (Worthley et al., 2015), *Gli1* (Shi et al., 2017), *LepR* (Zhou et al., 2014), *Pthrp* (Mizuhashi et al., 2018), *Sox9* (Kuwahara et al., 2019), *Cxcl12* (Matsushita et al., 2020), *Prx1* (Moore et al., 2018), etc. However, the proposed surface markers generally have the problem of low specificity, and as the physiological state and the microenvironment change, MSC markers will alter accordingly. With source diversity, MSCs can be isolated from hard tissues such as teeth, bone marrow, and craniofacial sutures. There is evidence that MSCs from different tissues are divergent (**Figure 1**) (Sharpe, 2016; Huang et al., 2019; Zhou et al., 2019). Although different tissue-derived MSC all meet the minimum standards for defining MSCs, their transcriptome pattern and multipotential differentiation capacity may also be vastly different (**Table 1**). Therefore, based on the complex expression alteration of MSC, a single surface marker is not enough to describe the heterogeneous MSC population.

## 3 SCRNA-SEQ-BASED MSC IDENTIFICATION AND FUNCTION ASSESSMENT

scRNA-seq profile gene expression at single-cell resolution, which is ideally suited to explore the heterogeneity of MSCs. The key to scRNA-seq technology is single-cell isolation and independent library construction. Using droplet-based microfluidics technology, single cell and gel beads containing a barcode, unique molecular index, primers, and enzymes were wrapped in an oil droplet through a microfluidic chip (Zhou et al., 2021). The barcode in each oil droplet is a unique DNA sequence, thus allowing the distinction of the source of the target sequence during sequencing (**Figure 2**). Therefore, researchers achieve large-scale single-cell isolation and routine profiling of thousands of cells via constructing libraries at one time. The scRNA-seq data set analysis of the MSC population roughly follows three main steps (Andrews et al., 2021). First, after quality control and normalization, the cells are divided into

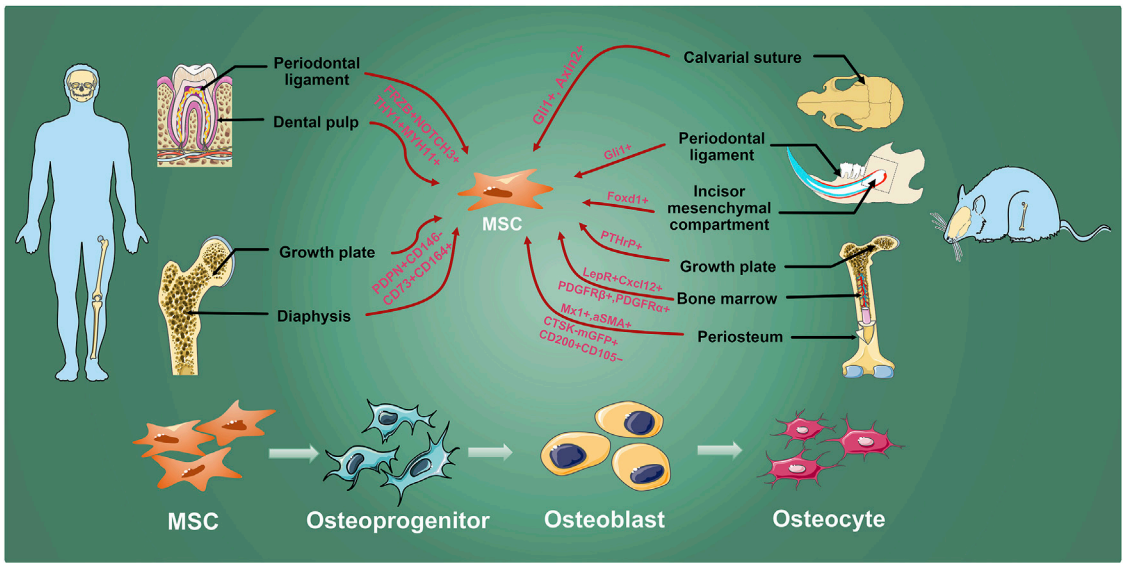


**FIGURE 1 |** MSC inhabit in various hard tissues. Osteogenic MSCs which are labeled by researchers with different genes exist in human molars and long bones, and mouse calvarial sutures, incisors, molars, and long bones. They are finally differentiated into osteocyte through osteoprogenitors and osteoblasts to involve in maintaining bone homeostasis, growth, development, and injury repair.

multiple subpopulations through dimensionality reduction and unsupervised clustering. Subpopulations are assigned cell types based on their gene expression patterns and prior knowledge. Second, analysis of cell heterogeneity within each cell type can identify MSC subpopulations with distinct cell states and expression programs. Genes that are differentially expressed between subpopulations can be regarded as potential markers. Third, among MSC-related studies, single-cell sequencing objects are typically a heterogeneous cell population, which emerges from the development or differentiation process of pluripotent stem cells making fate decisions and transitioning to specific cell types through intermediate cell states. Pseudotime inference constructs a pseudo-temporal process trajectory to order cells based on the gradual transition of transcriptomes. In MSC-related cases, these trajectories measure the relative progression of each cell along the development or differentiation process, allowing us to understand the pseudo-temporal behavior without explicit time series data (Campbell and Yau, 2018; Herring et al., 2018). Using scRNA-seq, researchers could subdivide MSCs population according to transcription information of individual cells, and predict potential gene markers to label cell populations by differential analysis, which is quite beneficial for finding suitable MSCs groups with different clinical needs of the stem cell therapy.

The recently emerging single-cell epigenome-, genome-, and proteome-sequencing technologies also provide promising directions (Nam et al., 2021). Li et al. (2018) team systematically describes the dynamic changes of the development of mouse preimplantation embryos from five epigenome levels (chromatin status, DNA methylation, copy number variation, and ploidy). Ai et al. (2019) and Wang et al. (2019) used independently developed CoBATCH and scitChIP technologies to profile the heterogeneity of endothelial lineage development, differentiation, and function in ten different tissues of mouse embryos. Chaligne et al. (2021) combined single-cell sequencing of DNA methylation, scRNA-seq and targeted genotyping to analyze diffuse gliomas, revealing dysregulated epigenetic mechanisms underlying gliomagenesis. Single-cell multi-omics integrated analysis is a powerful tool to study stem cell development, but there is still a gap in MSC-related research, and remains an uncovered space for future research.

## 3.1 Identification of MSCs in Embryonic and Fetal Limb Buds

In the initial stage of limb bud development, LMPs are composed of distinct progenitor cell types, which show heterogeneous characteristics and differentiated states (**Table 1**) (Norrie et al., 2014; Ornitz and Marie, 2015; Tickle and Towers, 2017). *SOX9*-expressing LMPs differentiate into cartilage and are regarded as osteochondrogenic progenitors (OCPs) (Akiyama et al., 2005), which generate primitive cartilage templates and then form limb bones through endochondral ossification. In contrast, another method of bone formation, intramembranous ossification builds the most of craniomaxillofacial bones. During this process, bone develops directly from sheets of mesenchymal tissue without the formation of intermediate cartilage.

In 2019, Reinhardt et al. (2019) identified the molecular characterization of various mesenchymal progenitor subpopulations in the early forelimb buds of mice (E10.5–E10.75). They found that *SOX9*$^{-}$*JAG1*$^{+}$ cells that resided in the distal posterior of mesenchyme were the most immature progenitor cells, which mainly depended on Sonic Hedgehog and apical ectodermal ridge-Fibroblast Growth Factor signaling

**TABLE 1 |** Characterization of embryonic and hard tissue-derived stem/progenitor cell (WPC, weeks post conception; yo, years old).

| Species | Localization | Age | Cell | Marker | Reference |
|---|---|---|---|---|---|
| Human | Limb buds | 5 WPC | Osteo-chondrogenic progenitorss | $SOX9^{low}PDGFR\alpha^{hi}$ | He et al. (2021a) |
| | Limb bud long bones | 8 WPC | Embryonic skeletal stem/progenitor cells | $PDGFR\alpha^{low/-}PDPN^{+}CADM1^{+}$ | He et al. (2021a) |
| | Embryonic calvarium | 8 WPC | Neural crest-derived cells | $PDGFR\alpha^{low/-}PDPN^{+}CADM1^{+}$ | He et al. (2021a) |
| | Third molar dental pulp and periodontal ligament | 18–35yo | Mesenchymal stem cells | $FRZB^{+}NOTCH3^{+}THY1^{+}$ $MYH11^{+}$ | Pagella et al. (2021) |
| | Femur growth plate and diaphysis | 17 weeks fetal | Human-skeletal stem cells | $PDPN^{+}CD146^{-}CD73^{+}CD164^{+}$ | Chan et al. (2018) |
| Mice | Forelimb buds | E10.5-E10.75 | Osteo-chondrogenic progenitors | $Sox9^{+}PDGFR\alpha^{hi}$ | Reinhardt et al. (2019) |
| | Forelimb buds | E10.5-E10.75 | Transition betweenlimb bud mesenchymal progenitors and osteo-chondrogenic progenitors | $Sox9^{-}PDGFR\alpha^{hi}$ | Reinhardt et al. (2019) |
| | Forelimb buds | E10.5-E10.75 | Limb bud mesenchymal progenitors | $Sox9^{-}JAG1^{+}$ | Reinhardt et al. (2019) |
| | Hind limb | E12.5 | Musculoskeletal stem cells | $Scx^{+}Hoxd13^{+}$ | Yin et al. (2020) |
| | Femur | Postnatal | Mouse skeletal stem cells | $CD45^{-}Ter119^{-}Tie^{-}AlphaV^{+}Thy^{-}6C3^{-}CD105^{-}CD200^{+}$ | Chan et al. (2015) |
| | Incisor mesenchymal compartment near the labial cervical loop | 2–4 months | Mesenchymal stem cells | $Foxd1^{+}$ | Krivanek et al. (2020) |
| | Molar periodontal ligament apical | Adult | Periodontal ligament stem cells | $Gli1^{+}$ | Men et al. (2020) |
| | The resting zone of growth plate | Postnatal | Skeletal stem cells | $PTHrP^{+}$ | Mizuhashi et al. (2018) |
| | The periphery of the growth plate immediately adjacent to the perichondrium | Fetal and neonatal | Mesenchymal precursor cells/ chondrocytes | $PTHrP^{+}$ | Mizuhashi et al. (2019) |
| | Metaphysis and diaphysis | 3 week | Mesenchymal stromal cells from the metaphysis and diaphysis | $PDGFR\alpha^{+}$, $PDGFR\beta^{+}$ | Sivaraj et al. (2021) |
| | Metaphysis and diaphysis | Postnatal | Bone marrow stromal cells | $LepR^{+}$ | Shu et al. (2021) |
| | Periosteum in the metaphysis and diaphysis | Adult | Periosteal skeletal stem cells | $Mx1^{+}$, $aSMA^{+}$ | Sivaraj et al. (2021) |
| | Bone marrow stromal of femur and tibia | 6–8 weeks | Mesenchymal stem cells | $LepR^{+}$ $Cxcl12^{+}$ | Baryawno et al. (2019) |
| | Long bone and calvarium | Postnatal | Periosteal stem cells | $CTSK\text{-}mGFP^{+}CD200^{+}CD105^{-}$ | Debnath et al. (2018) |

to maintain their proliferative and undifferentiated state, through *Grem1*-mediated Bone Morphogenetic Protein (BMP) antagonism to avoid BMP-induced apoptosis (Michos et al., 2004). In contrast, $SOX9^{+}PDGFR\alpha^{hi}$ OCPs are sensitive to BMP signaling in the mesenchyme core where limb buds will initially form, while changing from proliferative signals to responding to differentiation signaling. This kind of signal response transformation is a necessary condition for transformation from LMPs to OCPs. As a transition between $SOX9^{-}JAG1^{+}$ cells and $SOX9^{+}PDGFR\alpha^{hi}$ OCPs, $SOX9^{-}PDGFR\alpha^{hi}$ cells highly express T-Box transcription factor 2 (*TBX2*) which is involved in the repression of *Grem1*, enhancing the activity of BMP signals and endow LMPs with the potential to form limb buds (Michos et al., 2004; Farin et al., 2013). For murine hindlimb, Yin et al. (2020) named a cluster, composed predominantly of E12.5 cells which highly express scleraxis (*Scx*) and homeobox protein hox-D13 (*Hoxd13*) as musculoskeletal stem cells. $Scx^{+}$ musculoskeletal stem cells can generate soft tissue (myocytes, meniscus cells, and tenocytes) and hard tissue (chondrocytes and osteocytes) progenitors, marked by $Scx^{+}Col1a1^{+}$ and $Scx^{+}Sox9^{+}$ respectively. However, Kelly et al. (2020) observed faint and scattered expression of *Scx* at a time point after E13.5 and no expression in E11.5 and E13.5, and did not detect clusters marked by *Scx* from scRNA-seq analysis. The peak of *Scx* expression appeared in the middle of the trajectory (around E12.5). It has been reported, although *Scx* is transiently expressed in the chondrogenic lineage and entheseal cartilage, it is particularly important for the correct integration of musculoskeletal components (Blitz et al., 2013; Zelzer et al., 2014; Killian and Thomopoulos, 2016). Therefore, we speculated that *Scx*, a key transcription factor that regulates musculoskeletal tissue morphogenesis, may be only transiently expressed around E12.5, but the mechanism of *Scx* action remains unknown, and needs to be further investigated.

Human embryonic cells continuously differentiate from limb bud mesenchymal progenitor cells into OCPs, which gives rise to

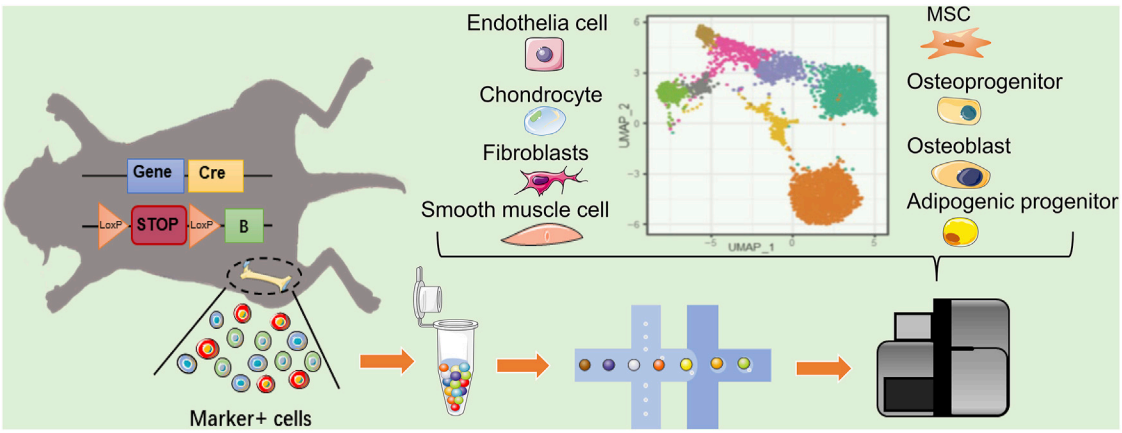


**FIGURE 2 |** Single cell reveals the heterogeneity of MSCs markers targeting cells. In mice with Cre–loxP recombination system, the MSC markers gene expressing-cells will be fluorescently labeled. When two loxP sites exist in the same DNA strand with the same orientation, terminator between two loxP will be removed by Cre, allowing the fluorescent protein to be expressed. The heterogeneity of labeled MSCs can revealed by scRNA-seq. In addition to MSC, the existing MSC marker-labeled cell population may include Osteo- and adipo-lineage cells, chondrocytes, epithelial cells, and fibroblast smooth muscle cells, etc. Blue square: MSC marker gene; yellow square: cyclization recombination enzyme gene; red rounded rectangle: stop codon; orange triangle: specific recognition site of Cre recombinase; green square: fluorescent reporter.

osteogenic and chondrogenic lineages. He et al. (2021a) identified four mesenchymal cell subpopulations (Limb Bud Mesenchyme1-3 and OCP) in human limb buds at 5 weeks post-conception (WPC). Similar to mouse OCPs, human limb bud OCPs (*SOX9*$^{low}$*PDGFRα*$^{hi}$) were located in the core mesenchyme and possessed chondrogenic potential. At 8WPC, a cluster marked by cell adhesion molecule 1 (*CADM1*) was assigned as perichondral embryonic skeletal stem/progenitor cells (eSSPCs); these cells are self-renewable and capable of generating osteochondral lineage. At the same developmental stage, a group of neural crest-derived stem/progenitor cells shared immunophenotype and transcriptional network as long bone-derived eSSPC were identified in the calvaria sagittal suture. They were characterized by the gene expression signature of intramembranous ossification, which mediates the development of the human craniofacial skeleton. In 2018, Chan et al. (2018) revealed markers of human SSC (*PDPN+CD146-CD73*$^{+}$*CD164+*) via scRNA-seq performed on a 17-week fetal femur growth plate and diaphysis. The isolated human SSCs displayed self-renewal abilities *in vitro* and could generate multilineage ossicles composed of bone, cartilage, and stromal progenitors under the renal capsule of NSG mice *in vivo*.

## 3.2 Hard Tissue-Derived MSCs in Postnatal Stages

Limb bones and MSCs residing in them primarily originate from the lateral plate mesoderm during embryogenesis, which create bone tissue by means of endochondral ossification. Neural crest cells located in the neuroectoderm are the principal source of craniofacial bones, cartilage and teeth. The neural crest-derived MSCs residing in the craniofacial region possess characteristics of multidirectional differentiation and low immunogenicity, which are comparable with the mesoderm-derived MSC, but the neural crest-derived MSCs are mostly involved in endochondral ossification.

### 3.2.1 Dental-Derived MSCs

Growing mouse incisors contains a continuously replenished mesenchymal compartment composed of dentin-secreting odontoblasts and various types of pulp cells. Krivanek et al. (2020) used single-cell technology to study the heterogeneity of the mesenchymal compartment in mouse incisors. They found a apical pulp subtype marked by *Smoc2* and *Sfrp2*, which was specifically located in the apical pulp area in cervical loops and included potential stem/progenitor cells that express genes related to self-renewal such as *Gli1* and *Thy1*. *Foxd1*$^{+}$ multipotent MSCs which mainly generated periodontoblastic pulp cells and odontoblasts were screened out, and *Foxd1*-traced cells were only detected in the mesenchymal compartment adhering to the labial cervical loop, revealing the pluripotency of *Foxd1*$^{+}$ MSCs and spatially constrained structure of the self-renew in growing mouse incisors. By comparing the cell subtypes of human apical and mouse distal incisor pulp, they inferred that although cell types are similar, evolutionary differences in gene expression programs that regulate development and homeostasis of dental pulp excluded the possibility of establishing precisely homologous subpopulation in the dental pulp between mouse and human (Krivanek et al., 2020). In adult mouse molar periodontal ligament, *Gli1*$^{+}$ cells enriched in the apical region are regarded as periodontal ligament stem cells, which can generate periodontal ligament, alveolar bone, and cementum, under Wnt signaling-mediated regulation (Men et al., 2020). Pagella et al. (2021) marked MSCs by highly expressed *FRZB*, *NOTCH3*,*THY1*, and *MYH11* in the dental pulp and periodontium of human third molars. In addition, *CCL2* and collagen-encoding genes were significantly higher in periodontal

MSCs than in dental pulp. In contrast, pulp MSCs expressed higher levels of *CXCL14* and *RARRES1* than periodontal MSCs. They revealed that the MSCs in the dental pulp and periodontium, both as neural crest-derived cells, shared a common phenotype, and contained stem cells with high regenerative potential, showing overall homology, which was consistent with the previous study (Luan et al., 2009). The specificity of the respective niche is a potential source of the divergence in MSC function, which direct periodontal and dental pulp MSC to fibroblastic-like and osteogenic fate, respectively.

### 3.2.2 Long Bone-Derived MSCs

The growth plate provides a continuous source of MSCs for endochondral ossification to construct a stromal compartment to maintain the expansion of bones and bone marrow space. *PTHrP*-expressing chondrocytes within the resting zone of the growth plate are considered one of the sources of SSCs, as they not only express a panel of skeletal stem/progenitor markers and possess characteristics of SSCs *in vitro* but also continuously form columnar chondrocytes that can generate osteoblasts and marrow stromal cells beneath the growth plate (Mizuhashi et al., 2018). In addition, the marginal chondrocytes around the growth plate behave as transient mesenchymal precursor cells, committed osteoblasts, and marrow stromal cells *in vivo*. Mizuhashi et al. (2019) performed single-cell sequencing on *Col2a1*-creER-marked chondrocytes in neonatal growth plates. They revealed that column-forming chondrocyte clusters from the growth plate of upper and lower region were marked by *Ucma* and *Prg4*, respectively; cluster abundant in tdTomato-WPRE was assigned as borderline chondrocytes between the upper and lower zone expressing *Pthrp* and *Cxcl14* and no hypertrophic markers. $PTHrP^+$ cells tracked non-self-renewing borderline chondrocyte subsets, which can give rise to short-lived osteoblasts and CXCL12-abundant reticular cells in the marrow cavity of long bone metaphysis (Mizuhashi et al., 2019).

MSCs in the periosteum are one of the major reservoirs of osteoprogenitor cells. Clinical and experimental data prove that the periosteum plays an essential role in postnatal bone growth, maintenance, and injury repair (Chaudhary et al., 2016; Debnath et al., 2018; Duchamp de Lageneste et al., 2018; Xiao et al., 2020). In adult mice, *Mx1*- and *aSMA*-labeled periosteal SSCs (P-SSCs) represent a unique lifelong sustainably regenerative stem cell group which serves as the main force for cortical bone regeneration and damage healing (Ortinau et al., 2019). There is evidence that P-SSCs contribute more to damage repair than bone marrow mesenchymal cells (BMSCs) (Worthley et al., 2015; Ortinau et al., 2019). With distinctive CCL5-dependent migration mechanism, $Mx1^+$ P-SSCs were rapidly recruited to injury area and generated osteoblasts, the number of which far exceeds of $Grem1^+$ bone marrow SSCs-derived (Ortinau et al., 2019). Besides, clusters of progenitor/stem cell and osteoblast were detected from scRNA-seq data of CTSK-mGFP+ periosteal mesenchymal cells. And in *Ctsk*-cre; mTmG reporter mice, Ctsk-cre-labeled $CD200^+CD105^-$ cells were regarded as periosteal MSCs (PSCs) because of their capacity for "trilineage" differentiation, self-renewal and generation of the entire spectrum of CTSK-$mGFP^+$ cells (bone, cartilage, stromal precursor/progenitor cells) (Debnath et al., 2018). PSCs specialize in intramembranous bone formation (Colnot, 2009), whereas P-SSCs display endochondral ossification and intramembranous bone formation.

BMSCs are the most commonly used stem cell in cell therapy clinical trials because of their ethical acceptability and accessibility. Researchers have characterized the heterogeneity and subpopulations of MSCs in different parts of the bone marrow cavity based on genetic fate tracking and single-cell sequencing (Severe et al., 2019; Tikhonova et al., 2019; Al-Sabah et al., 2020; Lu and Qiao, 2021). Baryawno et al. (2019) defined MSC clusters highly express *LepR* and *Cxcl12*, and two MSC-descendent osteolineage cells (OLC) subgroups express osteocalcin (*Bglap*). These two OLC subgroups are derived from distinct origins. The subpopulation composition in OLC-1 exhibited an osteolineage continuum from committed osteolineage LepR-MSC-4 in bone marrow, and expressed the key hematopoiesis-regulated cytokines, whereas OLC-2 mostly derived from bone, with no hematopoietic support potential. Besides, researchers have revealed temporal and spatial distinctions between BMSCs from the metaphysis (mpMSCs) and diaphysis (dpMSCs). Postnatal mpMSCs possessed multipotent properties. Clusters representing mpMSC, dpMSC, osteoprogenitor cells, osteoblasts, and proliferating BMSCs were identified in bone marrow. mpMSCs were placed in the center of pseudo-time trajectory, which directed to proliferating BMSC, dpMSC, and osteoprogenitor cell respectively (Sivaraj et al., 2021). $PDGFR\alpha^+\beta^+$ mpMSCs contained progenitors that gave rise to bone-forming osteoblast lineage cells, $LepR^+$ marrow stromal cells, and dpMSCs, whereas $PDGFR\alpha^+$ dpMSCs from juvenile mice showed limited growth *in vitro* (Sivaraj et al., 2021). In addition, Chondrocytes and $Lepr^+$ BMSCs mediate longitudinal growth and transverse thickening of the bone before and after adolescence, respectively. (Shu et al., 2021) reported that osteoblasts are mainly emerged from $Acan^+$ chondrocytes in the growth plate to realize bone lengthening before adolescence, whereas after adolescence, they primarily arise from $LepR^+$ BMSCs to achieve bone thickening.

### 3.2.3 Craniofacial Skeleton-Derived MSCs

In contrast to long bone endochondral ossification, the craniofacial skeleton is mainly formed through intramembranous ossification. As the main growth centers for craniofacial bone development, calvarial sutures preserve the population of MSCs that support craniofacial bone repair. $Gli1^+$ cells and $Axin2^+$ cells within the suture mesenchyme are the major MSC populations in the craniofacial bone and are regarded as suture stem cells (SuSCs) (Zhao et al., 2015; Maruyama et al., 2016). SuSCs are endowed with stem cell characteristics during calvarial development and homeostatic maintenance and are directly involved in injury repair and regeneration. Ablation of $Gli1^+$ and $Axin2^+$ cells leads to premature suture fusion (Zhao et al., 2015; Maruyama et al., 2016). However, the localization and responsible areas of $Axin2^+$ and $Gli1^+$ cells are different. $Gli1^+$ SuSCs are distributed throughout the mesenchyme suture and other osteogenesis regions within the suture, whereas *Axin2*-expressing cells are

**TABLE 2 |** Lineage-tracing mouse transgenic lines with stem/progenitor markers for scRNA-seq.

| Driver | Representative cre-marked cells | Single-cell sequencing object | Subclusters | Time points of induction | Reference |
|---|---|---|---|---|---|
| Gli1-CreERT2; tdTomato | Metaphyseal mesenchymal progenitor cells | TdTomato$^+$CD45$^-$Ter119$^-$CD31$^-$ cells | Osteoblasts, preosteoblasts, chondrocyte-like osteoprogenitors and marrow adipogenic lineage progenitors | Three consecutive days at 4 weeks old | Shi et al. (2021) |
| Pdgfrβ-CreERT2; Rosa26-mTmG | Pdgfrβ+BMSCs | GFP$^+$cells | BMSCs, chondrocytes, smooth muscle cells, fibroblasts, hematopoietic cells | Postnatal day 1–3 | Bohm et al. (2019) |
| Ctsk-cre; mTmG | Periosteal stem cells | Metaphyseal CTSK-mGFP$^+$ cells | Progenitor/stem cells, osteoblasts, *Ly6a* and *Acta2* expressing cells | N/A | Debnath et al. (2018) |
| Cxcl12GFP/+; Cxcl12-creER; R26RtdTomato | Cxcl12-creER+stromal cells | Cxcl12-GFP$^+$ cells | Stromal (reticular cells and pre-osteoblasts), endothelial, periosteal and cells in cell cycle, clusters enriched for mitochondrial and ribosomal genes | Postnatal day 21 | Matsushita et al. (2020) |
| Lepr-cre; LoxP-tdTomato | LepR+ cells | Lepr-tdT cells | Osteo-primed *LepR*$^+$ cells and adipocytic-primed *LepR*$^+$ cells | N/A | Tikhonova et al. (2019) |

only limited to the middle part of suture mesenchyme, almost not adjacent to bone tissues. In addition, calvarial sutures contain SuSCs with the same immunophenotype as long bone-derived PSCs and share the same intramembranous osteogenesis pathway as PSCs (Debnath et al., 2018). Recently, researchers have successfully used *Gli1*$^+$MSCs to regenerate a functional cranial suture and ameliorate craniosynostosis in a mouse model (Yu et al., 2021). Hence, SuSCs are expected to bring new vitality to human craniosynostosis therapy.

## 4 SCRNA-SEQ ON CRE-TARGETED MSCS IN TRANSGENIC MICE

Lineage tracing is important in stem cell research, which through the Cre recombinase (Cre)-*loxP* system permanently marks specific cells and tracks the proliferation, differentiation, and migration activities of specific cells and their descendants *in vivo*. Several endogenous osteogenesis stem/progenitor cell populations were marked by the expression of *Gli1, Osx, Ctsk, Pdgfrβ*, etc. In recent years, researchers have used lineage tracing combine with fluorescence-activated cell sorting techniques to tag and sort MSCs based on a series of markers, and subsequently performed scRNA-seq on them, thereby realizing the reconstruction of cell development trajectories and parsing the regulation of fate-determining gene expression (**Table 2**).

*Gli1*, which encodes the transcriptional key effector of Hedgehog (Hh), serves as a primary marker of MSCs. *Gli1*$^+$ cells continuously replenish osteoblasts for bone development and repair. Within craniofacial sutures, *Gli1*$^+$ cells were proposed as the major MSC population for craniofacial bone, responsible for the growth and injury repair. In postnatal mice, *Gli1*$^+$ cells inhabiting underneath the growth plate, were called metaphyseal mesenchymal progenitor cells, which can generate osteoblasts, adipocytes, and stromal cells *in vivo* and express *Pdgfrα*, *LepR*, and other MSC marker genes (Shi et al., 2017). *Gli1*$^+$ cells in the periodontal ligament of adult mouse molars can form the periodontal ligament, cementum, and alveolar bone. They support the renewal and injury repair of periodontal tissue, and their activity is modulated by canonical Wnt signaling (Men et al., 2020). In *Gli1*-CreERT2; tdTomato mice, metaphyseal mesenchymal progenitor cells (*Gli1*$^+$ cells) dissociated from metaphyseal trabecular bone, are clustered into four subsets: osteoblasts, pre-osteoblasts, chondrocyte-like osteoprogenitor, and marrow adipogenic lineage progenitors, among which chondrocyte-like osteoprogenitor is a potential target for widespread bone anabolic drug, teriparatide, and expresses the high levels of growth-related factors such as Hh target genes and *IGF-1* (Laron, 2001; Shi et al., 2021).

PDGFRβ is involved in the maintenance of immature and proliferative states for skeletal stem/progenitor cells (SSPCs). *Pdgfrβ*-traced cells were displayed in the growth plate, metaphyseal, periosteal, BMSCs, and perivascular space inhabited by osteo-primed multipotential stem cells (Bohm et al., 2019). In *Pdgfrb*-CreERT2; Rosa26-mTmG mice, long bone-derived GFP$^+$ cells belonged to three major sub-populations (BMSCs, chondrocytes, and smooth muscle cells) and two minor subsets (fibroblasts and hematopoietic cells). Trajectory analysis and *in vitro* culture of GFP$^+$ BMSC support *Pdgfrβ*$^+$ BMSCs possessing trilineage differentiation potential (Bohm et al., 2019). PDGFRβ promotes osteogenesis and angiogenesis in the postnatal period and activates SSPC in injury. Following a fracture, reparative SSPCs in the periosteal, endosteal and perivascular spaces were activated and recruited under the regulation of Pdgfrβ signaling. In *Pdgfrβ*$^+$ cell-ablated mice, the length of the femur and the number of Osx$^+$ osteoprogenitors decreased (Liu et al., 2013). In contrast, overexpression of human *Pdgfrβ* led to increased vessels, *Pdgfrβ*$^+$ mpMSCs, and osteoprogenitors (Bohm et al., 2019).

Osteoclasts secrete a cysteine protease called cathepsin K (CTSK) which plays an important role in the degradation of matrix collagen during bone resorption. Several studies have shown that *Ctsk*$^+$ cells possess progenitor/stem cell properties (Han et al., 2019). Yang et al. (2013) illustrated that chondroid neoplasms originate from the *Ctsk*-Cre labeled cell population in perichondrial groove of Ranvier, which exhibits markers and functional characteristics comparable to mesenchymal progenitors. Further studies have shown that *Ctsk*$^+$ periosteal

cells contain stem cell populations that mediate intramembranous osteogenesis. ScRNA-seq performed on *Ctsk*$^+$ cells derived from Ctsk-cre; mTmG reporter mouse femurs generated four clusters: expressing osteoblast markers, MSCs marker, stem cell antigen 1 (*Sca1*), and *Acta2*, respectively. Monocle-inferring differentiation trajectory showed that MSCs markers were expressed in the prophase of trajectory map containing periosteal stem cells, which supports the existence of stem/progenitor cells among *Ctsk*$^+$ cells (Debnath et al., 2018).

As a chemotactic protein, CXCL12 serves as an influential regulator of the musculoskeletal niche. It predominantly acts through the G-coupled protein receptor (CXCR4) and is involved in the conscription, location, growth, and fate determination of hematopoietic and MSCs in the musculoskeletal system (Wright et al., 2005; Hosogane et al., 2010; Fujio et al., 2011). Compared with immature MSCs, alkaline phosphatase-expressing differentiated osteogenic cells displayed decreased *Cxcl12* expression levels (Gilbert et al., 2019). CXCL12 is involved in the early differentiation of pre-osteoblast MSCs, but as the cell matures, the scale of involvement of CXCL12 gradually reduced. As a result, if CXCL12 signaling is disrupted, reduced migration and differentiation may ensue, potentially leading to a reduction in the quantity and viability of progenitor cells (Zhang et al., 2008; Guang et al., 2013). *CXCL12*-GFP$^+$ BM cells from *Cxcl12*$^{GFP/+}$; *CXCL12*-creER; R26RtdTomato mice were divided into three categories, stromal, endothelial, and periosteal at P28. *CXCL12*$^+$ stromal cell consisted of three clusters, two of which were reticular cells and osteogenic progenitors, expressing pre-adipocyte markers (*Adipoq* and *LepR*) and pre-osteoblast markers (*Alpl* and *Postn*), respectively. Upon the induction of injury, quiescent CXCL12-creER$^+$ BMSCs were recruited to the cortical defect, converted into SSC-like state through the regulation of canonical Wnt signaling components, and then differentiated into cortical bone, which does not occur in homeostasis (Matsushita et al., 2020).

As an endocrine hormone, leptin participates in energy metabolism and regulates the promotion and inhibition of MSCs osteogenesis. Previous studies have indicated that leptin enhances BMSC adipogenesis at the expense of osteogenesis, whereas it can promote osteogenesis of cultured MSC in other study (Thomas et al., 1999; Ambati et al., 2010). LepR is a marker that highly enriched BMSCs (Zhou et al., 2014). With trilineage differentiation potential, *LepR*-expressing cells can give rise to osteocytes, chondrocytes, and adipocytes through *in vitro* culture and xenotransplantation. Lineage tracing showed that *LepR*$^+$ cells are not only the main source of osteocytes and adipocytes in the adult bone marrow but also a cell reservoir of endochondral osteogenesis during embryonic and postnatal bone formation (Yue et al., 2016). Shen et al. (2021) revealed that an osteogenic growth factor, osteolectin, marked peri-arteriolar rapidly dividing *LepR*$^+$ osteogenic progenitor cells, which increased after injury and depleted during aging. During injury repair, *LepR*$^+$ cells form new chondrocytes and osteoblasts, whereas leptin inhibits bone regeneration by negatively regulating bone marrow osteogenesis (Zhou et al., 2014). *LepR*$^+$ MSCs labeled in LepR-cre; LoxP-tdTomato mice possessed potential for both adipogenesis and osteogenesis. The subpopulation clustered by *LepR*$^+$ cells scRNA-seq analysis showed high levels of adipogenesis- and osteogenesis-associated markers, respectively (Tikhonova et al., 2019). Furthermore, osteo-primed *LepR*$^+$ cells were highly correlated with multipotential human *CD45*$^-$*CD271*$^+$ BMSCs (Ghazanfari et al., 2016), and adipocytic-primed *LepR*$^+$ cells were the main pro-hematopoietic factor supplier in the BM niche. In response to stress hematopoiesis, adipogenesis-related pathways in *LepR*$^+$ cells significantly increased, and the expansion of adipocytes was observed after bone marrow insult. In contrast, the expression of ossification-related genes was decreased (Tikhonova et al., 2019).

Genetic lineage tracing based on Cre-loxP system is powerful and widely used in tracking stem cell differentiation and fate. However, single MSC markers are not specific to stem cell populations, making the reliability of this system still debatable. Dual-recombinase-activated lineage tracing (DeaLT) technology introduces the Dre-rox recombination system into the traditional Cre-loxP recombination system, allowing the more precise definition and tracking of cell populations, significantly improving the resolution of lineage fate maps. Different permutations and combinations of Dre-rox and Cre-loxP in the genome can produce different effects, mainly including the following three type: 1) Cre$^+$Dre$^+$, requires two promoters to drive Dre and Cre to recombination, mainly labeling the double-positive cells. 2) Cre$^+$Dre$^-$ or Cre$^-$Dre$^+$, highlight the exclusivity of the two recombination systems, mainly labeling single marker positive cells. 3) Cre$^+$Dre$^+$/Cre$^-$Dre$^+$ or Cre$^+$Dre$^+$/Cre$^+$Dre$^-$, mark the double-positive cells, and the cross-section is read out by one of the two markers. More detailed review information has been presented by Zhao and Zhou et al. (2019). This technology has been applied to reveal the developmental origin of hepatic vasculature and the cell fate of club cells, AT2 cells and bronchoalveolar stem cells in the process of lung repair and regeneration (Zhang et al., 2016; Liu et al., 2020). However, few MSC-related studies are using DeaLT technology (Shu et al., 2021). The combined application of DeaL technology and single-cell technology will bring more precise and reliable interpretations on the heterogeneity of MSCs, and facilitate understanding of the regulatory network that controls cell state, which exhibits extensive application prospects and warrant further exploration.

## 5 SINGLE-CELL ANALYSIS OF MSC IN RESPONSE TO MICROENVIRONMENT

The MSC niche supports the self-renewal and multi-lineage differentiation of MSCs. Distinct cell populations in the stem cell microenvironment provide signaling molecules for properly maintaining the proliferation and differentiation of stem cells, which are necessary for tissue stability. MSCs are heterogeneous, and the surrounding niche cells have different metabolic states. MSC will adapt to different microenvironments and perform specific functions through reprogramming. At present, with the help of single-cell sequencing technology, scientists can obtain gene expression profiles of MSC and surrounding niche cells, dissect the metabolic information of different cell types, evaluate

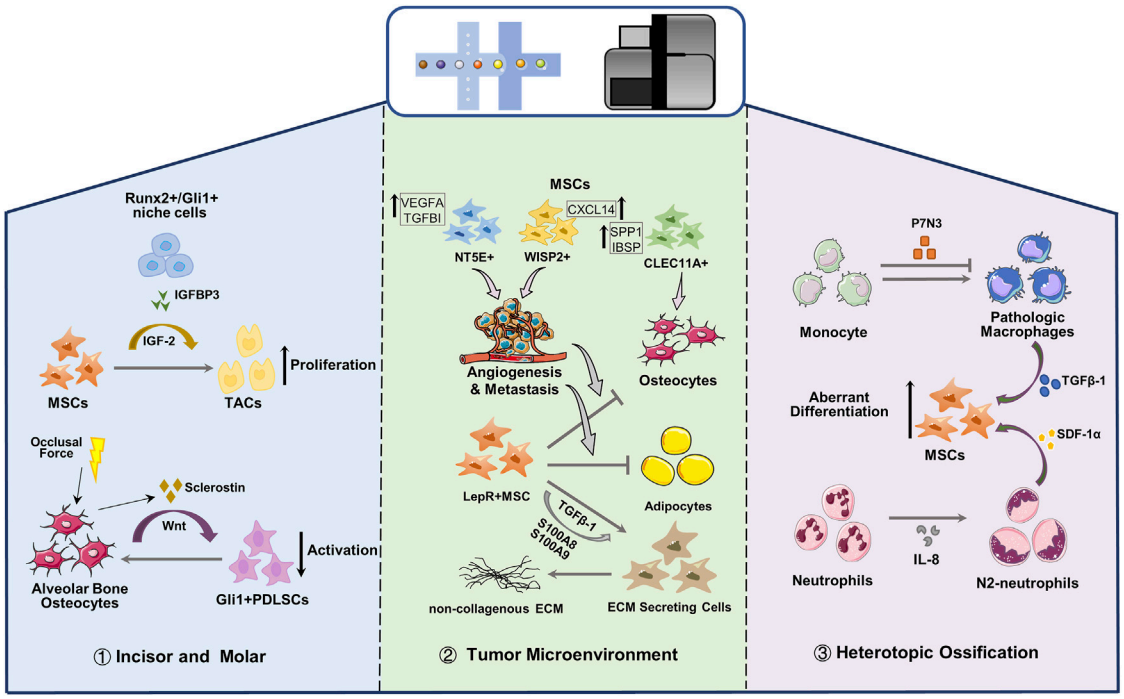


**FIGURE 3 |** Single cell reveals the crosstalk between MSCs and microenvironment. MSC, Mesenchymal stem cells; TAC, transit-amplifying cells; PDLSC, periodontal ligament stem cells; ECM, extracellular matrix.

the interaction between MSC and the microenvironment, and then study the genetic and environmental factors of MSC at the single-cell level.

## 5.1 Tooth Niche Cell Regulates MSCs Homeostasis

In the dental mesenchyme, *Gli1* is typical marker of MSCs in the mouse incisor and RUNX2 is an important transcription factor that regulates bone and tooth development. In adult mouse incisor, most *Gli1* and *Runx2* co-expressing cells were clustered into a subgroup located proximal region of dental mesenchyme and were adjacent to transit-amplifying cells, which suggested niche cell identity (Chen et al., 2020). Runx2$^+$/Gli1$^+$ niche cells secrete Insulin-like growth factor-binding protein 3, thereby activating the exp-mediated IGF2 signaling pathway and coordinating the transition of MSCs to transit-amplifying cells, thereby regulating the proliferation and differentiation of transit-amplifying cells, maintaining the homeostasis of mesenchymal tissue, and controlling the growth rate of incisors (Chen et al., 2020). In addition, studies have shown that osteogenesis in long bones or alveolar bone is governed by sclerostin and mechanical loading (Robling et al., 2008; Men et al., 2020). For the periodontal ligament, the activation and maintenance of *Gli1*$^+$ PDLSCs are modulated by canonical Wnt signaling, whereas alveolar bone cells that secrete sclerostin inhibit Wnt signaling and negatively modulate PDLSC activity (Men et al., 2020). Physiological occlusal force controlling the expression of sclerostin is indirectly involved in the activity of PDLSCs, and this mechano-response is essential for *Gli1*$^+$ PDLSCs activation (**Figure 3**).

## 5.2 The Lineage Transition of MSCs in Senescent

Aging is accompanied by the accumulation of genetic damage, leading to changes such as mutation, telomere shortening, cellular senescence, and stem cell depletion, etc. As the source of cells in bone marrow, MSC maintains bone metabolism. However, with the increase of age, MSCs tend to differentiate into adipocytes and decreased osteogenesis, leading to an increased risk of aging-related bone diseases for the elderly, and taking a long time to heal fractures (Woods and Guezguez, 2021). Experiments have shown that senescent MSCs exhibit reduced clonal formation and proliferation capabilities compared to young donor-derived MSCs *in vivo* and lineage tracing *in vitro* demonstrated an age-dependent lineage transition between osteogenesis and adipogenic differentiation (Sui et al., 2020). To define MSCs at young, adult, and aging stages, Zhong et al. (2020) performed single-cell sequencing of *Col2*$^+$ cells in the long bones of 1, 3, and 16-month-old mice, and divided the MSCs into three subgroups. Pseudotime trajectory inferred that the early mesenchymal progenitors (EMPs) in the three subgroups are the ancestors of two other subgroups expressing *Sca1*, *Thy1* and *Cd34*, and the next ones are the intermediate mesenchymal progenitor cells and late mesenchymal progenitors respectively. Consistent with the results of CFU-F, the number of MSCs with osteogenic/adipogenic capabilities in the bone marrow of aging mice

shrank sharply, while the number of adipocytes greatly increased. In young and adult mice, the intermediate and late mesenchymal progenitor clusters are concentrated at the starting point of differentiation. In the elderly, the loss of intermediate mesenchymal progenitor was observed. MSCs were prone to differentiation, and adipocyte markers such as *CBPA* and *LPL* were also highly expressed, which indicated that aged EMPs not only decrease in number but also drift towards a more adipogenic state. The expression of adult MSC marker *Lepr* in 16-month-old EMPs was significantly higher than that of 1 month, which was consistent with the previously observations that *Lepr*-Cre labeled MSCs only appeared in the bone marrow of adult mice, but not in young mice (Zhou et al., 2014; Zhong et al., 2020).

## 5.3 MSCs Under the Crosstalk With Tumor Microenvironment

Due to the aggressive growth of tumors, MSCs with regenerative capacity are recruited into lesions for tissue repair. In addition, the increased acidification, nutritional deficiency, inflammatory, hypoxia, and tumors microenvironment (TME) lead MSCs to accumulate in lesions. CXCR4 and Matrix Metalloproteinases 2 (MMP2) participate in the migration process of MSC to TME (Song and Li, 2011). After being recruited to the tumor, the crosstalk between MSCs and cancer cells reshapes the expression pattern of MSCs, thereby changing the direction of differentiation and adapting to the TME by acquiring certain functions, and this crosstalk also changes the metastatic potential and invasion efficiency of cancer cells (Whiteside, 2018). BMSCs promote breast carcinoma cells to produce lysyl oxidase, leading to increased metastatic potential and invasive activity of cancer cells (El-Haibi et al., 2012). In TME, MSCs are induced to differentiate into fibroblasts or myofibroblasts, which stabilize tumor tissue and enhance chemotherapy resistance and cancer stemness (Quante et al., 2011). TME-derived TGF-β1 activates MSCs to form cancer-associated fibroblasts-like phenotype. BMSC secrete a series of tumor suppressor factors such as DKK-1/3, interferon, CXCL10, IL12, etc. Dickkopf-1 (*DKK-1*) as a negative regulator of the Wnt/β-catenin pathway endow MSCs with anti-proliferation and anti-tumor effects on erythroleukemic and breast cancer cells (Thiago et al., 2010; Kim et al., 2015). BMSC derived-extracellular vesicles inhibit proliferation and promote apoptosis of liver carcinoma, Kaposi's sarcoma and ovarian tumor cell line (Takahara et al., 2016). The distinction between cancer-promoting MSCs and anti-tumor MSCs may be related to its heterogeneity in TME and the type of cancer. Therefore, it is necessary to explore the heterogeneity of MSC in TME.

Zhou et al. (2020) performed scRNA-seq on osteosarcoma lesions, and the identified clusters are all the progeny of MSC or hematopoietic stem cells. Malignant osteoblastic cells in osteosarcoma can be derived from any cell type in osteogenic lineage of MSCs. MSC cells characterized by *MMe*, *THY1* and *CXCL12* are divided into three subgroups ($NT5E^+$, $WISP2^+$ and $CLEC11A^+$), and are present in different types of osteosarcoma lesions (**Figure 3**). $NT5E^+$ MSCs is mainly observed in chondroblastic osteosarcoma lesions which stimulate the angiogenesis and metastasis of osteosarcoma cells; MSCs with high expression of *WISP* and *CXCL14* are regarded to promote the metastasis of osteosarcoma cells and the proliferation of MSCs; $CLEC11A^+$ MSCs mainly exist in osteoblast osteosarcoma lesions with highly expressing osteoblast differentiation markers (*SPP1* and *IBSP*). Although more efforts are required to reveal the influence of MSCs on malignant osteoblasts and chondrocytes in osteosarcoma lesions, gene expression data suggest that MSCs can help osteosarcoma cells to metastasize or proliferate (Zhou et al., 2020).

MSCs are the main members of the bone marrow niche, which can regulate hematopoietic stem cells and supply hormones and nutrition, and are closely related to various myeloid malignancies. The bone marrow niche under myeloid disease will reshape the transcription of MSCs, enabling lineage transfer and function reprogramming, and making it a potential target for the treatment of myeloid diseases. MSC has become a promising therapeutic tool for many clinical applications due to its unique immunomodulatory properties, which can secrete cytokines, reduce inflammation and cell apoptosis, and promote the proliferation of stem progenitor cells in endogenous tissues and organs (Sivanathan and Coates, 2018; Qin and Zhao, 2020; He et al., 2021b). In primary leukemia, the osteogenic Lepr-MSCs decreased, and the pre-osteoblasts in the OLC subgroup increased significantly (Baryawno et al., 2019). The osteogenic genes in Lepr-MSCs and OLC were down-regulated, and the genes that inhibit bone formation and calcification were up-regulated. The adipogenic genes in *Lepr*-MSCs were significantly down-regulated, resulting in the obstructed development of osteogenic lineage cells (**Figure 3**), the damaged adipocytes niche, and the loss of hematopoietic stem cells niche factors, leading to a niche that is not conducive to the production of normal blood cells (Baryawno et al., 2019). Besides, Adipogenic and osteogenic $Lepr^+$ MSCs are considered to be the main driving factors of myelofibrosis in myeloproliferative neoplasms (Koschmieder and Chatain, 2020). In Thrombopoietin-induced BM fibrosis, *LepR* and *Gli1* labeled adipogenic and osteogenic MSC clusters significantly up-regulate non-collagenous extracellular matrix (ECM) specific genes, and down-regulate MSC markers, which leaded to the loss of progenitor cell status and were reprogrammed into ECM secreting cells (**Figure 3**) (Leimkuhler et al., 2021). Expression profiles and pseudo-time trajectories showed that adipo- and osteo-primed MSC clusters exhibited high level adipogenic (*Adipoq*) and osteogenic (*Sp7*, *Ibsp*) signature, and were inferred to be pluripotent precursor cells. With the development of fibrosis, MSC marker genes and hematopoietic support genes were significantly down-regulated in all MSC clusters, while secreted factors (*S100A8*, *S100A9*) and genes related to ECM synthesis were significantly up-regulated, indicating that they lost the ability to support hematopoiesis and excessive maldifferentiation, resulting in exorbitant deposition of ECM in the bone marrow (Leimkuhler et al., 2021). MSC-mediated inflammation is the main driving force for the transition of the fibrotic lineage, especially S100A8/S100A9 pro-inflammatory factors expressed by MSCs and the

TGF-b signaling pathway. The novel small-molecule tumor suppressor, Tasquinimod, can inhibit S100A8/S100A9 signal transduction and effectively ameliorate fibrosis and the phenotype of myeloproliferative neoplasms (Leimkuhler et al., 2021).

### 5.4 Injury-Induced Niche Regulates MSC Behavior

In the musculoskeletal trauma site, inflammation niches recruit and orchestrate MSCs and immunocytes. MSCs attempt to regenerate tissues according to the prompts of inflammation and the immune microenvironment and sometimes lead to the abnormal cell fate of MSCs, resulting in heterotopic ossification (HO) (**Figure 3**). Fiber/adipogenic progenitor, a population of pluripotent stem cells, are the main contributors of HO, expressing PDGFRα and other MSC maker, which exhibits potential for adipogenic, chondrogenic and osteogenic differentiation (Lees-Shepard et al., 2018; Eisner et al., 2020). Fibrous/adipogenic progenitor cells can produce pivotal growth factors and matrix or matrisome proteins, such as IGF-1, TGF-β, collagens, integrins, etc., which impact the cellular physiology during injury, disease and homeostasis (Biferali et al., 2019; Theret et al., 2021). Recently, Sorkin et al. (2020) used single-cell transcriptome and trajectory analyses to identify different monocyte/macrophage subpopulations after injury and their dynamic changes in the different stages of inflammation after injury. They discovered a cluster of Pdgfrα labeled-stromal cells, and the abnormal differentiation of chondrogenic progenitor cell was predetermined 3 days after injury. After burn/tenotomy injury, p7N3 (transforming growth factor-1 expression regulator) treatment altered macrophage subset phenotypes. In addition, the expression levels of chondrogenic and osteogenic markers (*Sox9*, *Runx2*, *Acan* and *Col2a1*) of the stromal cell clusters in the p7N3 treatment groups are lower than control. They revealed that monocytes/macrophages expressing transforming growth factor-1 play an important role in endochondral osteogenesis-driven mesenchymal chondrogenic progenitor cell abnormal differentiation during HO progression. (Sorkin et al., 2020). Furthermore, Cai et al. (2021) found that a certain level of interleukin-8 (IL-8) polarizes neutrophils toward the N2 phenotype, which initiates bone regeneration by secreting stromal cell-derived factor-1α and initiating its downstream cascade reaction to mediate BMSC recruitment and differentiation, thereby inducing ectopic endochondral ossification.

## 6 CONCLUSION AND FUTURE PERSPECTIVES

In this review, we summarized the heterogeneity of human and mouse MSCs at single-cell resolution from embryo development to adulthood and emphasized subsets with osteogenic potential. During the initial stage of embryonic limb buds, the subpopulations of limb bud MSCs exhibit continuous marker expression patterns and uniquely respond to morphogenesis gradient signals and bone-forming signals, thereby displaying distinct differentiation states and osteogenic activity in different limb bud regions. Among them, subgroups marked by transient high expression marker genes require much weighted attention, in which phased transcription patterns in these subgroups have important consequences for musculoskeletal development. More nuanced analyses are needed to fully understand the changes in cell transcription during the development process, as well as the impact and significance of its existence. ScRNA-seq has identified stem/progenitor cell subsets with osteogenic potential in various cell types of hard tissues, including dental pulp/periodontal stem cells, bone marrow/periosteal stromal cells, chondrocytes, and craniofacial suture mesenchymal stem cells (Zhao et al., 2015; Mizuhashi et al., 2018; Tikhonova et al., 2019; Pagella et al., 2021). Unlike MSCs, SSCs identified in bone marrow have no adipogenic ability, and exclusively generate osteogenic lineages. Although many of the above-mentioned osteogenic cell subsets are called as MSCs, they may still retain adipogenic and myogenic potential. At present, the term, "skeletal stem cells", is under continuous refinement, the relationship and underlying molecular differences between SSCs and MSCs cannot be clearly defined yet. Similar to hematopoiesis, osteogenic stem cells also depend on the mediation of niche-derived signals, extracellular matrix, and cytokines. However, compared with the hematopoietic stem cell niche, studies on MSC microenvironment are relatively lacking. In addition to inflammatory microenvironment, the interaction between stem cells and surrounding niche cells is also largely unelucidated, when the crucial phase transitions of bone-forming pattern, especially during bone growth spurt in puberty and aging-related osteoporosis.

Besides scRNA-seq, single-cell technology includes single-cell ChIP-seq and ATAC-seq, etc., which allow the detection of intercellular heterogeneity and can comprehensively reveal interactions between cell movement, signal transduction pathways, transcription factors, and genome chromatin packaging, to deeply characterize stem cell populations in various tissues. Nonetheless, the main obstacle of single-cell technology is the loss of all the spatial information of the original cells. Recently emerging spatial transcriptome technology has swept the obstacle away, which allows the analysis of transcriptome information while preserving the spatial location of the tissue section (Liao et al., 2021). The integration of spatiotemporal information and single-cell transcriptomics pave the way for identifying the pivotal distinctions between cell subtypes and in-depth analysis of developmental tissues and is expected to bring new insights into MSCs.

## AUTHOR CONTRIBUTIONS

FS wrote the original manuscript and designed figures. YS designed the project and revised the article.

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## REFERENCES


Ai, S., Xiong, H., Li, C. C., Luo, Y., Shi, Q., Liu, Y., et al. (2019). Profiling Chromatin States Using Single-Cell itChIP-Seq. *Nat. Cel Biol.* 21, 1164–1172. doi:10.1038/s41556-019-0383-5

Akiyama, H., Kim, J.-E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., et al. (2005). Osteo-Chondroprogenitor Cells Are Derived from Sox9 Expressing Precursors. *Proc. Natl. Acad. Sci.* 102, 14665–14670. doi:10.1073/pnas.0504750102

Al-Sabah, J., Baccin, C., and Haas, S. (2020). Single-Cell and Spatial Transcriptomics Approaches of the Bone Marrow Microenvironment. *Curr. Opin. Oncol.* 32, 146–153. doi:10.1097/CCO.0000000000000602

Ambati, S., Li, Q., Rayalam, S., Hartzell, D. L., Della-Fera, M. A., Hamrick, M. W., et al. (2010). Central Leptin versus Ghrelin: Effects on Bone Marrow Adiposity and Gene Expression. *Endocrine* 37, 115–123. doi:10.1007/s12020-009-9274-z

Andrews, T. S., Kiselev, V. Y., McCarthy, D., and Hemberg, M. (2021). Tutorial: Guidelines for the Computational Analysis of Single-Cell RNA Sequencing Data. *Nat. Protoc.* 16, 1–9. doi:10.1038/s41596-020-00409-w

Baryawno, N., Przybylski, D., Kowalczyk, M. S., Kfoury, Y., Severe, N., Gustafsson, K., et al. (2019). A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia. *Cell* 177, 1915–1932. doi:10.1016/j.cell.2019.04.040

Biferali, B., Proietti, D., Mozzetta, C., and Madaro, L. (2019). Fibro-Adipogenic Progenitors Cross-Talk in Skeletal Muscle: The Social Network. *Front. Physiol.* 10, 1074. doi:10.3389/fphys.2019.01074

Blitz, E., Sharir, A., Akiyama, H., and Zelzer, E. (2013). Tendon-bone Attachment Unit Is Formed Modularly by a Distinct Pool of Scx- and Sox9-Positive Progenitors. *Development* 140, 2680–2690. doi:10.1242/dev.093906

Böhm, A.-M., Dirckx, N., Tower, R. J., Peredo, N., Vanuytven, S., Theunis, K., et al. (2019). Activation of Skeletal Stem and Progenitor Cells for Bone Regeneration Is Driven by PDGFRβ Signaling. *Develop. Cel* 51, 236–254. doi:10.1016/j.devcel.2019.08.013

Cai, B., Lin, D., Li, Y., Wang, L., Xie, J., Dai, T., et al. (2021). N2-Polarized Neutrophils Guide Bone Mesenchymal Stem Cell Recruitment and Initiate Bone Regeneration: A Missing Piece of the Bone Regeneration Puzzle. *Adv. Sci.* 8, e2100584. doi:10.1002/advs.202100584

Campbell, K. R., and Yau, C. (2018). Uncovering Pseudotemporal Trajectories with Covariates from Single Cell and Bulk Expression Data. *Nat. Commun.* 9, 2442. doi:10.1038/s41467-018-04696-6

Cao, Y., Buckels, E. J., and Matthews, B. G. (2020). Markers for Identification of Postnatal Skeletal Stem Cells *In Vivo*. *Curr. Osteoporos. Rep.* 18, 655–665. doi:10.1007/s11914-020-00622-2

Chaligne, R., Gaiti, F., Silverbush, D., Schiffman, J. S., Weisman, H. R., Kluegel, L., et al. (2021). Epigenetic Encoding, Heritability and Plasticity of Glioma Transcriptional Cell States. *Nat. Genet.* 53, 1469–1479. doi:10.1038/s41588-021-00927-7

Chan, C. K., Seo, E. Y., Chen, J. Y., Lo, D., McArdle, A., Sinha, R., et al. (2015). Identification and Specification of the Mouse Skeletal Stem Cell. *Cell* 160, 285–298. doi:10.1016/j.cell.2014.12.002

Chan, C. K. F., Gulati, G. S., Sinha, R., Tompkins, J. V., Lopez, M., Carter, A. C., et al. (2018). Identification of the Human Skeletal Stem Cell. *Cell* 175, 43–56. doi:10.1016/j.cell.2018.07.029

Chaudhary, R., Lee, M.-S., Mubyana, K., Duenwald-Kuehl, S., Johnson, L., Kaiser, J., et al. (2016). Advanced Quantitative Imaging and Biomechanical Analyses of Periosteal Fibers in Accelerated Bone Growth. *Bone* 92, 201–213. doi:10.1016/j.bone.2016.08.021

Chen, S., Jing, J., Yuan, Y., Feng, J., Han, X., Wen, Q., et al. (2020). Runx2+ Niche Cells Maintain Incisor Mesenchymal Tissue Homeostasis through IGF Signaling. *Cel Rep.* 32, 108007. doi:10.1016/j.celrep.2020.108007

Chen, Y., Yu, Q., Hu, Y., and Shi, Y. (2019). Current Research and Use of Mesenchymal Stem Cells in the Therapy of Autoimmune Diseases. *Curr. Stem Cell Res. Ther.* 14, 579–582. doi:10.2174/1574888X14666190429141421

Colnot, C. (2009). Skeletal Cell Fate Decisions within Periosteum and Bone Marrow during Bone Regeneration. *J. Bone Mineral Res.* 24, 274–282. doi:10.1359/jbmr.081003

Debnath, S., Yallowitz, A. R., McCormick, J., Lalani, S., Zhang, T., Xu, R., et al. (2018). Discovery of a Periosteal Stem Cell Mediating Intramembranous Bone Formation. *Nature* 562, 133–139. doi:10.1038/s41586-018-0554-8

Duchamp de Lageneste, O., Julien, A., Abou-Khalil, R., Frangi, G., Carvalho, C., Cagnard, N., et al. (2018). Periosteum Contains Skeletal Stem Cells with High Bone Regenerative Potential Controlled by Periostin. *Nat. Commun.* 9, 773. doi:10.1038/s41467-018-03124-z

Eisner, C., Cummings, M., Johnston, G., Tung, L. W., Groppa, E., Chang, C., et al. (2020). Murine Tissue-Resident PDGFRα+ Fibro-Adipogenic Progenitors Spontaneously Acquire Osteogenic Phenotype in an Altered Inflammatory Environment. *J. Bone Miner. Res.* 35, 1525–1534. doi:10.1002/jbmr.4020

El-Haibi, C. P., Bell, G. W., Zhang, J., Collmann, A. Y., Wood, D., Scherber, C. M., et al. (2012). Critical Role for Lysyl Oxidase in Mesenchymal Stem Cell-Driven Breast Cancer Malignancy. *Proc. Natl. Acad. Sci.* 109, 17460–17465. doi:10.1073/pnas.1206653109

Farin, H. F., Lüdtke, T. H.-W., Schmidt, M. K., Placzko, S., Schuster-Gossler, K., Petry, M., et al. (2013). Tbx2 Terminates Shh/Fgf Signaling in the Developing Mouse Limb Bud by Direct Repression of Gremlin1. *Plos Genet.* 9, e1003467. doi:10.1371/journal.pgen.1003467

Fujio, M., Yamamoto, A., Ando, Y., Shohara, R., Kinoshita, K., Kaneko, T., et al. (2011). Stromal Cell-Derived Factor-1 Enhances Distraction Osteogenesis-Mediated Skeletal Tissue Regeneration through the Recruitment of Endothelial Precursors. *Bone* 49, 693–700. doi:10.1016/j.bone.2011.06.024

Ghazanfari, R., Li, H., Zacharaki, D., Lim, H. C., and Scheding, S. (2016). Human Non-Hematopoietic CD271pos/CD140alow/neg Bone Marrow Stroma Cells Fulfill Stringent Stem Cell Criteria in Serial Transplantations. *Stem Cell Develop.* 25, 1652–1658. doi:10.1089/scd.2016.0169

Gilbert, W., Bragg, R., Elmansi, A. M., McGee-Lawrence, M. E., Isales, C. M., Hamrick, M. W., et al. (2019). Stromal Cell-Derived Factor-1 (CXCL12) and its Role in Bone and Muscle Biology. *Cytokine* 123, 154783. doi:10.1016/j.cyto.2019.154783

Guang, L. G., Boskey, A. L., and Zhu, W. (2013). Age-Related CXC Chemokine Receptor-4-Deficiency Impairs Osteogenic Differentiation Potency of Mouse Bone Marrow Mesenchymal Stromal Stem Cells. *Int. J. Biochem. Cel Biol.* 45, 1813–1820. doi:10.1016/j.biocel.2013.05.034

Han, Y., Feng, H., Sun, J., Liang, X., Wang, Z., Xing, W., et al. (2019). Lkb1 Deletion in Periosteal Mesenchymal Progenitors Induces Osteogenic Tumors through mTORC1 Activation. *J. Clin. Invest.* 129, 1895–1909. doi:10.1172/JCI124590

He, J., Yan, J., Wang, J., Zhao, L., Xin, Q., Zeng, Y., et al. (2021a). Dissecting Human Embryonic Skeletal Stem Cell Ontogeny by Single-Cell Transcriptomic and Functional Analyses. *Cell Res* 31, 742–757. doi:10.1038/s41422-021-00467-z

He, J., Zhang, N., Zhu, Y., Jin, R., and Wu, F. (2021b). MSC Spheroids-Loaded Collagen Hydrogels Simultaneously Promote Neuronal Differentiation and Suppress Inflammatory Reaction through PI3K-Akt Signaling Pathway. *Biomaterials* 265, 120448. doi:10.1016/j.biomaterials.2020.120448

Herring, C. A., Chen, B., McKinley, E. T., and Lau, K. S. (2018). Single-Cell Computational Strategies for Lineage Reconstruction in Tissue Systems. *Cell Mol. Gastroenterol. Hepatol.* 5, 539–548. doi:10.1016/j.jcmgh.2018.01.023

Hosogane, N., Huang, Z., Rawlins, B. A., Liu, X., Boachie-Adjei, O., Boskey, A. L., et al. (2010). Stromal Derived Factor-1 Regulates Bone Morphogenetic Protein 2-Induced Osteogenic Differentiation of Primary Mesenchymal Stem Cells. *Int. J. Biochem. Cel Biol.* 42, 1132–1141. doi:10.1016/j.biocel.2010.03.020

Huang, Y., Li, Q., Zhang, K., Hu, M., Wang, Y., Du, L., et al. (2019). Single Cell Transcriptomic Analysis of Human Mesenchymal Stem Cells Reveals Limited Heterogeneity. *Cell Death Dis* 10, 368. doi:10.1038/s41419-019-1583-4

Kelly, N. H., Huynh, N. P. T., and Guilak, F. (2020). Single Cell RNA-Sequencing Reveals Cellular Heterogeneity and Trajectories of Lineage Specification During Murine Embryonic Limb Development. *Matrix Biol.* 89, 1–10. doi:10.1016/j.matbio.2019.12.004

Killian, M. L., and Thomopoulos, S. (2016). Scleraxis Is Required for the Development of a Functional Tendon Enthesis. *FASEB j.* 30, 301–311. doi:10.1096/fj.14-258236

Kim, H. Y., Park, J. H., Won, H. Y., Lee, J. Y., and Kong, G. (2015). CBX7 Inhibits Breast Tumorigenicity through DKK-1-mediated Suppression of the Wnt/β-Catenin Pathway. *FASEB j.* 29, 300–313. doi:10.1096/fj.14-253997

Koschmieder, S., and Chatain, N. (2020). Role of Inflammation in the Biology of Myeloproliferative Neoplasms. *Blood Rev.* 42, 100711. doi:10.1016/j.blre.2020.100711

Krivanek, J., Soldatov, R. A., Kastriti, M. E., Chontorotzea, T., Herdina, A. N., Petersen, J., et al. (2020). Dental Cell Type Atlas Reveals Stem and

Differentiated Cell Types in Mouse and Human Teeth. *Nat. Commun.* 11, 4816. doi:10.1038/s41467-020-18512-7

Kuwahara, S. T., Serowoky, M. A., Vakhshori, V., Tripuraneni, N., Hegde, N. V., Lieberman, J. R., et al. (2019). Sox9+ Messenger Cells Orchestrate Large-Scale Skeletal Regeneration in the Mammalian Rib. *eLife* 8, e40715. doi:10.7554/eLife.40715

Laron, Z. (2001). Insulin-like Growth Factor 1 (IGF-1): a Growth Hormone. *J. Clin. Pathol.-Mol. Pathol.* 54, 311–316. doi:10.1136/mp.54.5.311

Lees-Shepard, J. B., Yamamoto, M., Biswas, A. A., Stoessel, S. J., Nicholas, S.-A. E., Cogswell, C. A., et al. (2018). Activin-Dependent Signaling in Fibro/adipogenic Progenitors Causes Fibrodysplasia Ossificans Progressiva. *Nat. Commun.* 9, 471. doi:10.1038/s41467-018-02872-2

Leimkühler, N. B., Gleitz, H. F. E., Ronghui, L., Snoeren, I. A. M., Fuchs, S. N. R., Nagai, J. S., et al. (2021). Heterogeneous Bone-Marrow Stromal Progenitors Drive Myelofibrosis via a Druggable Alarmin Axis. *Cell Stem Cell* 28, 637–652. doi:10.1016/j.stem.2020.11.004

Li, L., Guo, F., Gao, Y., Ren, Y., Yuan, P., Yan, L., et al. (2018). Single-cell Multi-Omics Sequencing of Human Early Embryos. *Nat. Cel Biol.* 20, 847–858. doi:10.1038/s41556-018-0123-2

Lian, J. B., Stein, G. S., Javed, A., van Wijnen, A. J., Stein, J. L., Montecino, M., et al. (2006). Networks and Hubs for the Transcriptional Control of Osteoblastogenesis. *Rev. Endocr. Metab. Disord.* 7, 1–16. doi:10.1007/s11154-006-9001-5

Liao, J., Lu, X., Shao, X., Zhu, L., and Fan, X. (2021). Uncovering an Organ's Molecular Architecture at Single-Cell Resolution by Spatially Resolved Transcriptomics. *Trends Biotechnol.* 39, 43–58. doi:10.1016/j.tibtech.2020.05.006

Liu, K., Tang, M., Jin, H., Liu, Q., He, L., Zhu, H., et al. (2020). Triple-Cell Lineage Tracing by a Dual Reporter on a Single Allele. *J. Biol. Chem.* 295, 690–700. doi:10.1074/jbc.RA119.011349

Liu, Y., Strecker, S., Wang, L., Kronenberg, M. S., Wang, W., Rowe, D. W., et al. (2013). Osterix-Cre Labeled Progenitor Cells Contribute to the Formation and Maintenance of the Bone Marrow Stroma. *PLoS One* 8, e71318. doi:10.1371/journal.pone.0071318

Lu, S., and Qiao, X. (2021). Single-Cell Profiles of Human Bone Marrow-Derived Mesenchymal Stromal Cells after IFN-γ and TNF-α Licensing. *Gene* 771, 145347. doi:10.1016/j.gene.2020.145347

Luan, X., Dangaria, S., Ito, Y., Walker, C. G., Jin, T., Schmidt, M. K., et al. (2009). Neural Crest Lineage Segregation: A Blueprint for Periodontal Regeneration. *J. Dent. Res.* 88, 781–791. doi:10.1177/0022034509340641

Maes, C., Kobayashi, T., Selig, M. K., Torrekens, S., Roth, S. I., Mackem, S., et al. (2010). Osteoblast Precursors, but Not Mature Osteoblasts, Move into Developing and Fractured Bones along with Invading Blood Vessels. *Develop. Cel* 19, 329–344. doi:10.1016/j.devcel.2010.07.010

Maruyama, T., Jeong, J., Sheu, T.-J., and Hsu, W. (2016). Stem Cells of the Suture Mesenchyme in Craniofacial Bone Development, Repair and Regeneration. *Nat. Commun.* 7, 10526. doi:10.1038/ncomms10526

Matsushita, Y., Nagata, M., Kozloff, K. M., Welch, J. D., Mizuhashi, K., Tokavanich, N., et al. (2020). A Wnt-Mediated Transformation of the Bone Marrow Stromal Cell Identity Orchestrates Skeletal Regeneration. *Nat. Commun.* 11, 332. doi:10.1038/s41467-019-14029-w

Men, Y., Wang, Y., Yi, Y., Jing, D., Luo, W., Shen, B., et al. (2020). Gli1+ Periodontium Stem Cells Are Regulated by Osteocytes and Occlusal Force. *Develop. Cel* 54, 639–654. doi:10.1016/j.devcel.2020.06.006

Michos, O., Panman, L., Vintersten, K., Beier, K., Zeller, R., and Zuniga, A. (2004). Gremlin-mediated BMP Antagonism Induces the Epithelial-Mesenchymal Feedback Signaling Controlling Metanephric Kidney and Limb Organogenesis. *Development* 131, 3401–3410. doi:10.1242/dev.01251

Mizuhashi, K., Nagata, M., Matsushita, Y., Ono, W., and Ono, N. (2019). Growth Plate Borderline Chondrocytes Behave as Transient Mesenchymal Precursor Cells. *J. Bone Miner. Res.* 34, 1387–1392. doi:10.1002/jbmr.3719

Mizuhashi, K., Ono, W., Matsushita, Y., Sakagami, N., Takahashi, A., Saunders, T. L., et al. (2018). Resting Zone of the Growth Plate Houses a Unique Class of Skeletal Stem Cells. *Nature* 563, 254–258. doi:10.1038/s41586-018-0662-5

Moore, E. R., Yang, Y., and Jacobs, C. R. (2018). Primary Cilia Are Necessary for Prx1-Expressing Cells to Contribute to Postnatal Skeletogenesis. *J. Cel Sci.* 131, jcs217828. doi:10.1242/jcs.217828

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., et al. (2002). The Novel Zinc Finger-Containing Transcription Factor Osterix Is Required for Osteoblast Differentiation and Bone Formation. *Cell* 108, 17–29. doi:10.1016/S0092-8674(01)00622-5

Nam, A. S., Chaligne, R., and Landau, D. A. (2021). Integrating Genetic and Non-Genetic Determinants of Cancer Evolution by Single-Cell Multi-Omics. *Nat. Rev. Genet.* 22, 3–18. doi:10.1038/s41576-020-0265-5

Norrie, J. L., Lewandowski, J. P., Bouldin, C. M., Amarnath, S., Li, Q., Vokes, M. S., et al. (2014). Dynamics of BMP Signaling in Limb Bud Mesenchyme and Polydactyly. *Develop. Biol.* 393, 270–281. doi:10.1016/j.ydbio.2014.07.003

Ono, N., Ono, W., Mizoguchi, T., Nagasawa, T., Frenette, P. S., and Kronenberg, H. M. (2014). Vasculature-Associated Cells Expressing Nestin in Developing Bones Encompass Early Cells in the Osteoblast and Endothelial Lineage. *Develop. Cel* 29, 330–339. doi:10.1016/j.devcel.2014.03.014

Ornitz, D. M., and Marie, P. J. (2015). Fibroblast Growth Factor Signaling in Skeletal Development and Disease. *Genes Dev.* 29, 1463–1486. doi:10.1101/gad.266551.115

Ortinau, L. C., Wang, H., Lei, K., Deveza, L., Jeong, Y., Hara, Y., et al. (2019). Identification of Functionally Distinct Mx1+αSMA+ Periosteal Skeletal Stem Cells. *Cell Stem Cell* 25, 784–796. doi:10.1016/j.stem.2019.11.003

Pagella, P., de Vargas Roditi, L., Stadlinger, B., Moor, A. E., and Mitsiadis, T. A. (2021). A Single-Cell Atlas of Human Teeth. *iScience* 24, 102405. doi:10.1016/j.isci.2021.102405

Ponte, F., Kim, H. N., Iyer, S., Han, L., Almeida, M., and Manolagas, S. C. (2020). Cxcl12 Deletion in Mesenchymal Cells Increases Bone Turnover and Attenuates the Loss of Cortical Bone Caused by Estrogen Deficiency in Mice. *J. Bone Miner. Res.* 35, 1441–1451. doi:10.1002/jbmr.4002

Qin, H., and Zhao, A. (2020). Mesenchymal Stem Cell Therapy for Acute Respiratory Distress Syndrome: from Basic to Clinics. *Protein Cell* 11, 707–722. doi:10.1007/s13238-020-00738-2

Quante, M., Tu, S. P., Tomita, H., Gonda, T., Wang, S. S. W., Takashi, S., et al. (2011). Bone Marrow-Derived Myofibroblasts Contribute to the Mesenchymal Stem Cell Niche and Promote Tumor Growth. *Cancer Cell* 19, 257–272. doi:10.1016/j.ccr.2011.01.020

Ramos, T. L., Sánchez-Abarca, L. I., Muntión, S., Preciado, S., Puig, N., López-Ruano, G., et al. (2016). MSC Surface Markers (CD44, CD73, and CD90) Can Identify Human MSC-Derived Extracellular Vesicles by Conventional Flow Cytometry. *Cell Commun. Signal.* 14, 2. doi:10.1186/s12964-015-0124-8

Reinhardt, R., Gullotta, F., Nusspaumer, G., Ünal, E., Ivanek, R., Zuniga, A., et al. (2019). Molecular Signatures Identify Immature Mesenchymal Progenitors in Early Mouse Limb Buds that Respond Differentially to Morphogen Signaling. *Development* 146, dev173328. doi:10.1242/dev.173328

Robling, A. G., Niziolek, P. J., Baldridge, L. A., Condon, K. W., Allen, M. R., Alam, I., et al. (2008). Mechanical Stimulation of Bone *In Vivo* Reduces Osteocyte Expression of Sost/Sclerostin. *J. Biol. Chem.* 283, 5866–5875. doi:10.1074/jbc.M705092200

Severe, N., Karabacak, N. M., Gustafsson, K., Baryawno, N., Courties, G., Kfoury, Y., et al. (2019). Stress-Induced Changes in Bone Marrow Stromal Cell Populations Revealed through Single-Cell Protein Expression Mapping. *Cell Stem Cell* 25, 570–583. e7. doi:10.1016/j.stem.2019.06.003

Sharpe, P. T. (2016). Dental Mesenchymal Stem Cells. *Development* 143, 2273–2280. doi:10.1242/dev.134189

Shen, B., Tasdogan, A., Ubellacker, J. M., Zhang, J., Nosyreva, E. D., Du, L., et al. (2021). A Mechanosensitive Peri-Arteriolar Niche for Osteogenesis and Lymphopoiesis. *Nature* 591, 438–444. doi:10.1038/s41586-021-03298-5

Shi, Y., He, G., Lee, W.-C., McKenzie, J. A., Silva, M. J., and Long, F. (2017). Gli1 Identifies Osteogenic Progenitors for Bone Formation and Fracture Repair. *Nat. Commun.* 8, 2043. doi:10.1038/s41467-017-02171-2

Shi, Y., Liao, X., Long, J. Y., Yao, L., Chen, J., Yin, B., et al. (2021). Gli1+ Progenitors Mediate Bone Anabolic Function of Teriparatide via Hh and Igf Signaling. *Cel Rep.* 36, 109542. doi:10.1016/j.celrep.2021.109542

Shu, H. S., Liu, Y. L., Tang, X. T., Zhang, X. S., Zhou, B., Zou, W., et al. (2021). Tracing the Skeletal Progenitor Transition during Postnatal Bone Formation. *Cell Stem Cell* 28, 2122–2136. doi:10.1016/j.stem.2021.08.010

Sivanathan, K. N., and Coates, P. T. (2018). IL-17A-Induced Mesenchymal Stem Cells Have Promising Therapeutic Value for Clinical Translation. *Kidney Int.* 93, 771–773. doi:10.1016/j.kint.2017.12.010

Sivaraj, K. K., Jeong, H.-W., Dharmalingam, B., Zeuschner, D., Adams, S., Potente, M., et al. (2021). Regional Specialization and Fate Specification of Bone Stromal Cells in Skeletal Development. *Cel Rep.* 36, 109352. doi:10.1016/j.celrep.2021.109352

Song, C., and Li, G. (2011). CXCR4 and Matrix Metalloproteinase-2 Are Involved in Mesenchymal Stromal Cell Homing and Engraftment to Tumors. *Cytotherapy* 13, 549–561. doi:10.3109/14653249.2010.542457

Sorkin, M., Huber, A. K., Hwang, C., Carson, W. F., Menon, R., Li, J., et al. (2020). Regulation of Heterotopic Ossification by Monocytes in a Mouse Model of Aberrant Wound Healing. *Nat. Commun.* 11, 722. doi:10.1038/s41467-019-14172-4

Sui, B.-D., Zheng, C.-X., Li, M., Jin, Y., and Hu, C.-H. (2020). Epigenetic Regulation of Mesenchymal Stem Cell Homeostasis. *Trends Cel Biol.* 30, 97–116. doi:10.1016/j.tcb.2019.11.006

Takahara, K., Ii, M., Inamoto, T., Nakagawa, T., Ibuki, N., Yoshikawa, Y., et al. (2016). microRNA-145 Mediates the Inhibitory Effect of Adipose Tissue-Derived Stromal Cells on Prostate Cancer. *Stem Cell Develop.* 25, 1290–1298. doi:10.1089/scd.2016.0093

Theret, M., Rossi, F. M. V., and Contreras, O. (2021). Evolving Roles of Muscle-Resident Fibro-Adipogenic Progenitors in Health, Regeneration, Neuromuscular Disorders, and Aging. *Front. Physiol.* 12, 673404. doi:10.3389/fphys.2021.673404

Thiago, L. S., Costa, E. S., Lopes, D. V., Otazu, I. B., Nowill, A. E., Mendes, F. A., et al. (2010). The Wnt Signaling Pathway Regulates Nalm-16 B-Cell Precursor Acute Lymphoblastic Leukemic Cell Line Survival and Etoposide Resistance. *Biomed. Pharmacother.* 64, 63–72. doi:10.1016/j.biopha.2009.09.005

Thomas, T., Gori, F., Khosla, S., Jensen, M. D., Burguera, B., and Riggs, B. L. (1999). Leptin Acts on Human Marrow Stromal Cells to Enhance Differentiation to Osteoblasts and to Inhibit Differentiation to Adipocytes1. *Endocrinology* 140, 1630–1638. doi:10.1210/endo.140.4.6637

Tickle, C., and Towers, M. (2017). Sonic Hedgehog Signaling in Limb Development. *Front. Cel Dev. Biol.* 5, 14. doi:10.3389/fcell.2017.00014

Tikhonova, A. N., Dolgalev, I., Hu, H., Sivaraj, K. K., Hoxha, E., Cuesta-Domínguez, Á., et al. (2019). The Bone Marrow Microenvironment at Single-Cell Resolution. *Nature* 569, 222–228. doi:10.1038/s41586-019-1104-8

Uccelli, A., Moretta, L., and Pistoia, V. (2008). Mesenchymal Stem Cells in Health and Disease. *Nat. Rev. Immunol.* 8, 726–736. doi:10.1038/nri2395

Wang, Q., Xiong, H., Ai, S., Yu, X., Liu, Y., Zhang, J., et al. (2019). CoBATCH for High-Throughput Single-Cell Epigenomic Profiling. *Mol. Cel* 76, 206–216. doi:10.1016/j.molcel.2019.07.015

Wei, Y., Sun, H., Gui, T., Yao, L., Zhong, L., Yu, W., et al. (2021). The Critical Role of Hedgehog-Responsive Mesenchymal Progenitors in Meniscus Development and Injury Repair. *Elife* 10, e62917. doi:10.7554/eLife.62917

Whiteside, T. L. (2018). Exosome and Mesenchymal Stem Cell Cross-Talk in the Tumor Microenvironment. *Semin. Immunol.* 35, 69–79. doi:10.1016/j.smim.2017.12.003

Woods, K., and Guezguez, B. (2021). Dynamic Changes of the Bone Marrow Niche: Mesenchymal Stromal Cells and Their Progeny during Aging and Leukemia. *Front. Cel Dev. Biol.* 9, 714716. doi:10.3389/fcell.2021.714716

Worthley, D. L., Churchill, M., Compton, J. T., Tailor, Y., Rao, M., Si, Y., et al. (2015). Gremlin 1 Identifies a Skeletal Stem Cell with Bone, Cartilage, and Reticular Stromal Potential. *Cell* 160, 269–284. doi:10.1016/j.cell.2014.11.042

Wright, L., Maloney, W., Yu, X., Kindle, L., Collinosdoby, P., and Osdoby, P. (2005). Stromal Cell-Derived Factor-1 Binding to its Chemokine Receptor CXCR4 on Precursor Cells Promotes the Chemotactic Recruitment, Development and Survival of Human Osteoclasts. *Bone* 36, 840–853. doi:10.1016/j.bone.2005.01.021

Xiao, H., Wang, L., Zhang, T., Chen, C., Chen, H., Li, S., et al. (2020). Periosteum Progenitors Could Stimulate Bone Regeneration in Aged Murine Bone Defect Model. *J. Cel. Mol. Med.* 24, 12199–12210. doi:10.1111/jcmm.15891

Yang, W., Wang, J., Moore, D. C., Liang, H., Dooner, M., Wu, Q., et al. (2013). Ptpn11 Deletion in a Novel Progenitor Causes Metachondromatosis by Inducing Hedgehog Signalling. *Nature* 499, 491–495. doi:10.1038/nature12396

Yin, Z., Lin, J., Yan, R., Liu, R., Liu, M., Zhou, B., et al. (2020). Atlas of Musculoskeletal Stem Cells with the Soft and Hard Tissue Differentiation Architecture. *Adv. Sci.* 7, 2000938. doi:10.1002/advs.202000938

Yu, M., Ma, L., Yuan, Y., Ye, X., Montagne, A., He, J., et al. (2021). Cranial Suture Regeneration Mitigates Skull and Neurocognitive Defects in Craniosynostosis. *Cell* 184, 243–256. doi:10.1016/j.cell.2020.11.037

Yue, R., Zhou, B. O., Shimada, I. S., Zhao, Z., and Morrison, S. J. (2016). Leptin Receptor Promotes Adipogenesis and Reduces Osteogenesis by Regulating Mesenchymal Stromal Cells in Adult Bone Marrow. *Cell Stem Cell* 18, 782–796. doi:10.1016/j.stem.2016.02.015

Zelzer, E., Blitz, E., Killian, M. L., and Thomopoulos, S. (2014). Tendon-to-Bone Attachment: From Development to Maturity. *Birth Defect Res. C* 102, 101–112. doi:10.1002/bdrc.21056

Zhang, H., Pu, W., Tian, X., Huang, X., He, L., Liu, Q., et al. (2016). Genetic Lineage Tracing Identifies Endocardial Origin of Liver Vasculature. *Nat. Genet.* 48, 537–543. doi:10.1038/ng.3536

Zhang, W., Dong, Z., Li, D., Li, B., Liu, Y., Zheng, X., et al. (2021). Cathepsin K Deficiency Promotes Alveolar Bone Regeneration by Promoting Jaw Bone Marrow Mesenchymal Stem Cells Proliferation and Differentiation via Glycolysis Pathway. *Cell Prolif* 54, e13058. doi:10.1111/cpr.13058

Zhang, W., Ou, G., Hamrick, M., Hill, W., Borke, J., Wenger, K., et al. (2008). Age-Related Changes in the Osteogenic Differentiation Potential of Mouse Bone Marrow Stromal Cells. *J. Bone Miner. Res.* 23, 1118–1128. doi:10.1359/jbmr.080304

Zhao, H., Feng, J., Ho, T.-V., Grimes, W., Urata, M., and Chai, Y. (2015). The Suture Provides a Niche for Mesenchymal Stem Cells of Craniofacial Bones. *Nat. Cel Biol.* 17, 386–396. doi:10.1038/ncb3139

Zhao, H., and Zhou, B. (2019). Dual Genetic Approaches for Deciphering Cell Fate Plasticity *In Vivo*: More Than Double. *Curr. Opin. Cel Biol.* 61, 101–109. doi:10.1016/j.ceb.2019.07.004

Zhong, L., Yao, L., Tower, R. J., Wei, Y., Miao, Z., Park, J., et al. (2020). Single Cell Transcriptomics Identifies a Unique Adipose Lineage Cell Population that Regulates Bone Marrow Environment. *eLife* 9, e54695. doi:10.7554/eLife.54695

Zhou, B. O., Yue, R., Murphy, M. M., Peyer, J. G., and Morrison, S. J. (2014). Leptin-Receptor-Expressing Mesenchymal Stromal Cells Represent the Main Source of Bone Formed by Adult Bone Marrow. *Cell Stem Cell* 15, 154–168. doi:10.1016/j.stem.2014.06.008

Zhou, W.-M., Yan, Y.-Y., Guo, Q.-R., Ji, H., Wang, H., Xu, T.-T., et al. (2021). Microfluidics Applications for High-Throughput Single Cell Sequencing. *J. Nanobiotechnol.* 19, 312. doi:10.1186/s12951-021-01045-6

Zhou, W., Lin, J., Zhao, K., Jin, K., He, Q., Hu, Y., et al. (2019). Single-Cell Profiles and Clinically Useful Properties of Human Mesenchymal Stem Cells of Adipose and Bone Marrow Origin. *Am. J. Sports Med.* 47, 1722–1733. doi:10.1177/0363546519848678

Zhou, Y., Yang, D., Yang, Q., Lv, X., Huang, W., Zhou, Z., et al. (2020). Single-Cell RNA Landscape of Intratumoral Heterogeneity and Immunosuppressive Microenvironment in Advanced Osteosarcoma. *Nat. Commun.* 11, 6322. doi:10.1038/s41467-020-20059-6

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REVIEW
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# Stem Cell-Based Therapy for Diabetic Foot Ulcers

*Qian Yu[1†], Guo-hong Qiao[2†], Min Wang[3], Li Yu[3], Yaoxiang Sun[2], Hui Shi[2,3*] and Tie-liang Ma[2*]*

[1]Department of Hepatology, Songjiang Hospital Affiliated to Nanjing Medical University, Shanghai, China, [2]Department of Clinical Laboratory, The Affiliated Yixing Hospital of Jiangsu University, Yixing, China, [3]Jiangsu Key Laboratory of Medical Science and Laboratory Medicine, Institute of Stem Cell, School of Medicine, Jiangsu University, Zhenjiang, China

Diabetic foot ulcer has become a worldwide clinical medical challenge as traditional treatments are not effective enough to reduce the amputation rate. Therefore, it is of great social significance to deeply study the pathogenesis and biological characteristics of the diabetic foot, explore new treatment strategies and promote their application. Stem cell-based therapy holds tremendous promise in the field of regenerative medicine, and its mechanisms include promoting angiogenesis, ameliorating neuroischemia and inflammation, and promoting collagen deposition. Studying the specific molecular mechanisms of stem cell therapy for diabetic foot has an important role and practical clinical significance in maximizing the repair properties of stem cells. In addition, effective application modalities are also crucial in order to improve the survival and viability of stem cells at the wound site. In this paper, we reviewed the specific molecular mechanisms of stem cell therapy for diabetic foot and the extended applications of stem cells in recent years, with the aim of contributing to the development of stem cell-based therapy in the repair of diabetic foot ulcers.

**Keywords: diabetic foot ulcers, stem cell therapy, angiogenesis, anti-inflammatory, diabetic neuropathy**

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*Gianandrea Pasquinelli,*
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*Sherif Yakout Shalaby,*
*Sefako Makgatho Health Sciences University, South Africa*

****Correspondence:***
*Hui Shi*
*shihui@ujs.edu.cn*
*Tie-liang Ma*
*staff1762@yxph.com*

*†These authors have contributed equally to this work*

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## INTRODUCTION

Diabetic foot is the most common complication of diabetes and is prone to recurrence and infection. If this occurs in individuals who have an impaired immune function, the wound will become a portal to infection, leading to the presence of sepsis and amputation (Heublein et al., 2015), which is highly correlated with increased mortality. 85% of lower limb amputations are caused by diabetic foot ulcers (DFUs) (Boulton, 2010). In the absence of effective interventions, many DFU patients have experienced multiple amputations throughout their lives. Compared with people without diabetes, diabetic patients have 15 times higher amputation rates of lower limbs (Nicolucci et al., 2006). Chronic ulcers are difficult to cure, prone to recurrence, and have a high rate of amputation, significantly reducing the patients' quality of life. It is a source of psychological burden and economic pressure for patients and places a substantial financial burden on their families and the health care system in general. Therefore, there is an unmet clinical need for new approaches to accelerate diabetic wound healing, especially in comorbidities (e.g., lower extremity ischemic disease, cardiovascular disease).

The traditional treatment of diabetic foot is mainly medical treatment and surgical blood flow reconstruction. However, for foot ischemia caused by arterial stenosis and occlusion, medical treatment cannot fundamentally solve the problem. Surgical treatment as an effective method to restore blood flow reconstruction also faces some problems. For example, the lower extremity artery

lesions in diabetic foot patients mostly involve the lower leg artery (Qin et al., 2016), and many patients lack the distal arterial outflow tract (Houlind, 2020). These patients often face the risk of amputation due to the inability to receive arterial bypass or interventional therapy. Furthermore, patients with diabetic foot are often accompanied by cardiovascular and cerebrovascular diseases, so they cannot withstand the irritation of distal bypass surgery.

Lately, efforts have been made to develop innovative and effective therapies to repair chronic wounds, including topical application of growth factors or cell-based therapies. Becaplermin, a recombinant platelet-derived growth factor, is the only drug approved by the FDA to treat diabetic neuropathic ulcers. It has similar biological activities to endogenous platelet-derived growth factors, including improving chemotactic recruitment of cells involved in wound repair, promoting cell proliferation as well as angiogenesis, and enhancing granulation tissue formation (Regranex, 2008). However, the use of Becaplermin is facing several problems, including low systemic bioavailability and malignant tumors far from the application site. Besides, it is not clear whether it is valid for diabetic ischemic ulcers. Therefore, although growth factors have been shown to play an essential role in DFUs, it is necessary to explore new treatments to deal with the possible adverse consequences of diabetic foot. In addition, it is now possible to enhance the delivery efficiency of growth factors through cellular therapy (Gauglitz and Jeschke, 2011).

Although both growth factors and stem cells have defects in repairing diabetic foot, compared with the direct application of growth factors, the main advantage of stem cells in diabetic foot is that stem cells can regulate tissue regeneration in an all-around way by improving the microenvironment at the wound site. The individual use of single cytokines or growth factors obviously oversimplifies the complexity of the wound healing process involved.

Stem cells are the critical cells in post-injury and routine homeostasis skin repair. In recent years, stem cell transplantation has attracted more and more attention as a new technique for treating diabetic lower limb ischemic disease, including the diabetic foot. Stem cell therapy aims to stimulate the formation of new blood vessels to increase blood supply and relieve limb ischemia, ultimately promoting wound healing. Moreover, the administration of stem cells based on traditional treatment undoubtedly better exploit the role of stem cells in repairing damage and greatly improves the negative consequences of severe complications of diabetic foot. For example, angioplasty combined with human umbilical cord mesenchymal stem cells (hUC-MSCs) transplantation can improve the blood supply of severe diabetic foot, promote ulcer healing, reduce amputation rate and mortality, and improve the quality of life of patients with advanced diabetic foot (Qin et al., 2016). In addition, there is evidence that the application of myogenic mesenchymal stem cells (MMSCs) can essentially reverse the vascular occlusion of diabetes-related peripheral artery disease (PAD) (Hedhli et al., 2017).

In contrast to this eminent function of stem cells, however, the mechanisms underlying an impaired wound healing process are poorly understood. Therefore, a better understanding of the characteristics of stem cells and the signals that control their behavior is expected to bring new hope to the treatment of the diabetic foot. This review will begin with an overview of the characteristics of the diabetic foot, followed by an overview of stem cells and their therapeutic potential and our current understanding of extended application of stem cell-based therapy in DFUs.

## CHARACTERISTICS OF THE DIABETIC FOOTS

Diabetic foot presents a long-term complex interaction of neuropathic, macrovascular, and microvascular diseases in an abnormal metabolic context, accompanied by a decline in healing ability. Diabetes can cause microvascular dysfunction, and as a result, the microvascular supply to the leg nerves may be affected, resulting in abnormal foot movements (motor nerves), paresthesia (sensory nerves), and decreased sweating (autonomic nerves) (Cavanagh et al., 2005; Grennan, 2019). Asymptomatic diabetic peripheral neuropathy is up to 50% (American Diabetes Association, 2016). Even if patients are symptomatic, less than a third of physicians can recognize the manifestations of peripheral neuropathy associated with diabetes (International Diabetes Federation, 2019). Early symptoms (pain and paresthesia) of diabetic peripheral neuropathy are caused by small fibers, while large fiber lesions cause numbness and loss of protective sensation (LOPS) (Forbes and Cooper, 2013; American Diabetes Association, 2016). Due to LOPS, patients are unaware of minor injuries due to external trauma and/or foot deformity (Steed et al., 2006; Vileikyte et al., 2017). Therefore, these ulcers may have enlarged before they are detected, and by the time they are detected, 25–50% of the foot ulcers already have gangrene (Lepäntalo et al., 2011; Grennan, 2019). Due to some of the above possible reasons, diabetic foot ulcers have become the most ordinary complication of diabetes-related complications. Its lifetime incidence is estimated at 19–34% (Armstrong et al., 2017). The incidence of recurrence of foot ulcers within 5 years is up to 65% (Armstrong et al., 2017).

Although diabetic peripheral neuropathy is a microvascular complication, it may adversely affect microvascular function (Stirban, 2014; Barwick et al., 2016). It has been reported that ischemic ulcers may account for only 10% of diabetic foot lesions, and 90% are caused by neuropathy, alone or in association with ischemia (Boulton, 2010). Diabetic foot is ischemic, and the large blood vessels in the legs may also be affected by diabetes (Grennan, 2019). Compared with lower limb ischemia caused by simple arteriosclerosis, the treatment of diabetes combined with lower limb arterial ischemia is more difficult and complicated. Poor artery flow will reduce blood supply to the ulcer areas, ultimately resulting in impaired oxygen delivery and nutritional supply (Steed et al., 2006; Jiang et al., 2012). These effects may slow the healing of the ulcers. Peripheral nerve injury combined with reduced blood flow perfusion (including large and microvascular vessels) increases the likelihood of foot ulceration, infection, and eventual amputation (Lepäntalo et al., 2011; Bus, 2012). Also, high blood sugar can

delay wound healing and worsen the infection (van Crevel et al., 2017; Giri et al., 2018; Grennan, 2019).

At the molecular and cellular levels, abnormalities of cytokines produced by inflammatory cells, decreased macrophage infiltration, reduced growth factor, reduced collagen synthesis and impaired neovascularization make wound healing slow or nonhealing. The interaction of various complex factors eventually makes the wound repair process abnormal, from blood hemostasis and coagulation to inflammation, then epithelialization as well as angiogenesis, and finally granulation tissue formation. Therefore, compared with routine wound healing, the diabetic ulcer has prominent edema and bleeding, dermal or epidermal tissue formation is obstructed, granulation tissue formation is significantly reduced, matrix maturation and remodeling are incomplete, and finally, the loose and irregular connective tissue is formed (Lee et al., 2011).

## OVERVIEW OF STEM CELLS AND THEIR THERAPEUTIC POTENTIAL

Stem cells have the characteristics of asymmetric replication, the potential of strong self-renewal, and multi-differentiation (Ding et al., 2011). Asymmetric replication describes the unique properties of stem cells: stem cells undergo mitosis to produce progeny (self-renewal) with the same properties as their mothers and progeny (differentiated cells) with more restrictive potential (Lanza and Atala, 2014).

Embryonic stem cells (ESCs) can be obtained from the inner cell mass of the blastocyst early in development (Bogliotti et al., 2018) or prepared *in vitro* by *in vitro* fertilization (Hikabe et al., 2016), or the nuclei of somatic cells can be transplanted into the enucleated oocytes using cloning techniques and isolated from the cells of their inner cell mass when they reach the blastocyst stage of development (Hochedlinger and Jaenisch, 2003). Despite their extraordinary potential, the use of ESCs in the clinical setting remains limited. First, the use of hESCs is highly ethically controversial. Although ESCs can be obtained without destroying the embryos, it is unclear whether this approach necessarily eliminates ethical issues and whether sufficient ESCs can be generated in this method. In addition, ESCs may not be safe; they are inclined to have a vigorous proliferative capacity and lower differentiation maturity, so the introduction of these cells may cause immune rejection and stimulation of tumor formation. Therefore, ESCs are rarely employed for the treatment of DFUs (Jiang et al., 2012).

Adult stem cells are not ethically controversial and can be isolated from several sources. They were initially found in the bone marrow but are currently thought to exist in almost every organ, including fat, umbilical cord, and placenta (Ding et al., 2011). They have multi-directional differentiation potential and can be induced to differentiate into at least three different types of functional cells, even across germ layers, which is a reversal of the traditional understanding that adult stem cells can only differentiate into functional cells of the corresponding germ layer (Lanza and Atala, 2014). Although adult stem cells are less potent, they are still significant for maintaining overall health.

Nerves, which were previously thought to be non-regenerative, have now been found to be regeneratively repairable through stem cell differentiation (Mathot et al., 2019). By injecting stem cells into the patients' muscles or arteries or applying them directly to the wound locally, stem cells can be localized to the injured tissue by some mechanism and promoted epidermal regeneration by secreting cytokines to promote neovascularization. In addition, coldness and pain caused by nerve ischemia may be relieved (Wang et al., 2019).

Different types of stem cells can improve chronic diabetic wounds by enhancing the expression levels of vascular endothelial growth factor (VEGF), promoting cell proliferation, angiogenesis, and granulation tissue formation in traumatic tissues (Lee et al., 2011; Wan et al., 2013). In addition, inflammation and immune regulation also play an essential role in promoting wound healing (Wetzler et al., 2000; Loretelli et al., 2020). Stem cells have been shown in experiments to successfully cure DFUs, offering new prospects for the development of effective therapies for clinical use. Currently, 40 clinical trials have been found on a search for "diabetic foot ulcers, stem cell" (Clinicaltrials.gov, 2021).

## MOLECULAR MECHANISMS TO REPAIRING DFUS

### Repairing Diabetic Foot by Promoting Angiogenesis

Due to ethical constraints, the research of ESCs in wound repair is limited to a certain extent. However, some animal experiments have shown that ESCs have broad prospects in repairing chronic wounds, especially during the early stage of wound repair. Lee et al. (2011) found in the experiment of topical application of ESCs on the full-thickness skin of diabetes-induced rats that the expression levels of fibronectin, VEGF, and epidermal growth factor (EGF) are significantly higher than those of the insulin-treated diabetic group.

Currently, mesenchymal stem cells (MSCs)-based methods have been proposed as prospective therapies for delayed or impaired healing wounds, and numerous studies have shown that MSCs can promote diabetic wound healing (**Table 1**). Experiments show that intramuscular transplantation works best (Wan et al., 2013). MSCs can systematically mobilize and find home for injured and ischemic tissues, creating a wound microenvironment conducive to wound healing and ultimately promote the wound healing process of diabetic foot (Zhang et al., 2010; Wan et al., 2013; Shi et al., 2016; Shi et al., 2020) (**Figure 1**). Wound healing is a complex and multifactorial tissue regeneration process involving the interaction between epidermal and dermal cells, as well as the interaction between cells and cytokines. For example, Shi et al. (2020) detected increased levels of VEGF, basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) in DFUs treated with UC-MSCs. HGF, a downstream pathway of PI3K, can upregulate VEGF expression through the c-MET signaling pathway (Matsumura et al., 2013). TNF-α, as an essential component at the wound site, could secret VEGF and HGF by

**TABLE 1 |** Potential role of stem cells in the healing process of DFUs.

| References | Cell type | Objective | Mode of administration | Changes in the molecular level | Changes in histology/Clinical manifestation |
|---|---|---|---|---|---|
| Lee et al. (2011) | ESCs | STZ-induced T1DM in Sprague-Dawley rats | Topical application | EGF and VEGF↑, fibronectin levels↑ | High vascular counts, moderate re-epithelialization, well-formed granulation tissue formation, and capillary vessels |
| Loretelli et al. (2020) | ESCs | Db/db mice* | Topical application of EXTs | Tregs↑, proliferating Ki-67$^+$ cells↑, CD31$^+$ endothelial cells↑, CD45$^+$ inflammatory cells↓, IFN-γ↓, Th1↓ | Re-epithelialization, angiogenesis, and reduced leukocyte infiltration |
| Wan et al. (2013) | BM-MSCs | STZ-induced T1DM in Wistar rats | Intramuscular transplantation | VEGF↑, proliferating Ki-67$^+$ cells↑, CD31$^+$ endothelial cells↑ | Angiogenesis, cellular proliferation, and granulation tissue formation |
| Kuo et al. (2011) | BM-MSCs | STZ-induced T1DM in Wistar rats | Subdermal injection | CD45↓, VEGF↑, EGF↑, prolyl 4-hydroxylase↑, Ki-67 expression↑ | Proinflammatory reaction↓, wound sizes↓ |
| Dash et al. (2009), Wang et al. (2019) | BM-MSCs | 24/4 diabetic patients | Intramuscular transplantation | — | Accelerate the healing process, pain relief, wound size↓ |
| Kato et al. (2014) | BM-MSCs | STZ-induced T1DM in Sprague-Dawley rats | Topical application | Expression levels of MMP-2, EGF, IGF-1, and pFAK↑, the expression level of MMP-9↓ | Wound size↓ |
| Uchiyama et al. (2017) | BM-MSCs | Db/db mice; WT mice; and C57BL/6 mice | Intradermal injection | IL-10↑, TNF-α↓, the number of M2 macrophages↑ | Promote angiogenesis and accelerate diabetic wound healing |
| Lv et al. (2017) | BM-MSCs/stem cells from human SHED | STZ-induced T1DM in Sprague-Dawley rats | Topical application | Expression levels of VEGF, eNOS, MMP-2 and MMP9↑, the expression of IL-1β, IL-10, and TNF-1α↓ | Inflammatory cells↓, epithelialization↑, well formation of granulation tissue |
| Zhao et al. (2013) | hUC-MSCs | STZ-induced T1DM in Sprague-Dawley rats | Left femoral artery injection | Cytokeratin 19↑, collagen I and collagen III↑, the ratio of collagen I/III↓ | — |
| Xia et al. (2015) | hUC-MSCs | STZ-induced T1DM in Sprague-Dawley rats | Left femoral artery injection | Serum NGF↑ | Numbers of capillary↑ |
| Yue et al. (2020) | hUC-MSCs | STZ-induced T1DM in Sprague-Dawley rats | Subcutaneous injection | Expression levels of PDGFA and HGF↑ | Accelerate diabetic wound healing, angiogenesis, and re-epithelialization |
| Shi et al. (2020) | hUC-MSCs | STZ-induced type 1 diabetes in Sprague-Dawley rats | Transfemoral vein transplantation | Ki-67 + cells↑, VEGF, bFGF, and HGF↑, IL-1ra, IL-10, CINC-1, CINC-2α/β, CINC-3, CNTF, CCL3, CCL5, CCL20, CX3CL1, CXCL7, and LIX↑, IL13↓ | Collagen deposition and vascular density↑, closely complete re-epithelialization, fewer infiltrating inflammatory cells, and thick granulation tissue |
| Li et al. (2013) | hUC-MSCs | 15 diabetic patients | Intramuscular injection | The ratios of Treg/Th17 and Treg/Th1 cells↑, VEGF↑, IL-6↓ | — |
| Zhang S et al. (2020) | UCMSCs | STZ-induced C57BL/6J female mice | Subcutaneous injection | IL-10↑, IL-1β, TNF-α, and IL-6↓, the number of M2 macrophages↑ | Skin angiogenesis |
| Shi et al. (2016) | HADSCs | STZ-induced type 1 diabetes in Sprague-Dawley rats | Transfemoral vein transplantation | IL-1ra, IL-2, TNF-α, and CNTF↓, IL-1β, IL-6, IL-13, CCL3, CINC-1, CINC-2α/β, CINC 3, CX3CL1, LECAM-1, and LIX↑, VEGF, bFGF, and TGF-β↑ | Epithelialization↑, well formation of granulation tissue |
| De Gregorio et al. (2020) | HADSCs | Db/db mice* | Systemic administration | Expression of IL-1β and TNF-1α↑ | Reducing chronic inflammation of peripheral nerves and improving angiogenesis |

(Continued on following page)

**TABLE 1 |** (*Continued*) Potential role of stem cells in the healing process of DFUs.

| References | Cell type | Objective | Mode of administration | Changes in the molecular level | Changes in histology/Clinical manifestation |
|---|---|---|---|---|---|
| Uzun et al. (2020) | HADSCs | 20 patients | Dermo-epidermal junction injection | — | Wound size↓ |
| Maharlooei et al. (2011) | HADSCs | STZ-induced type 1 diabetes in Sprague-Dawley rats | Subdermal injection | — | Wound size↓ |
| An et al. (2020) | HADSCs | STZ-induced C57BL/6J male mice | Topical application | Collagen I and collagen III↑, IL-6↓ | Skin angiogenesis |
| Wang et al. (2016) | PMSCs | GK rats† | Subcutaneous injection | TNF-α, IL-6 and IL-1↓, IL-10↑ | Angiogenesis, collagen deposition and thick granulation tissue, the infiltration of macrophage↓ |
| Zhao et al. (2020a) | Combined use of ECFCs, HA, and UBC-MSCs | 12 subjects | Topical cell injection | — | Re-epithelialization rate↑, wound size↓ |
| Yang et al. (2020a) | Combination product of dermal matrix, timolol (beta-adrenergic antagonist), and BM-MSCs | Db/db mice* | Scaffold's implantation | CD31 + cells↑, CD45 + cells↓, CCL2 expression level↑, IL-1β, IL-6, CXCL-1, and CXCL-2↓ | Anti-inflammatory and pro-angiogenic functions↑ |

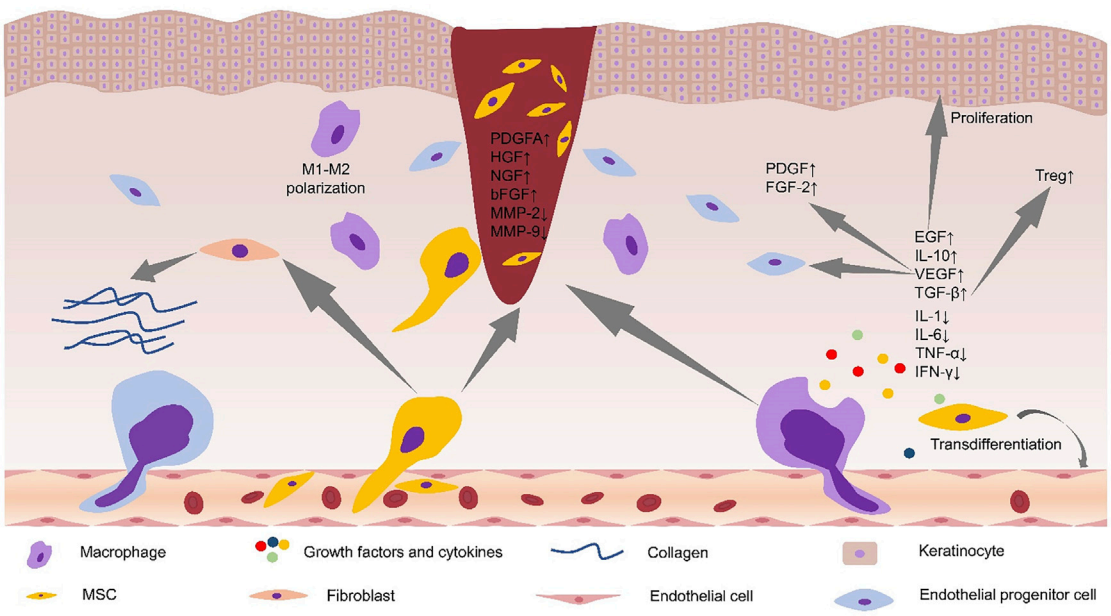


**FIGURE 1 |** Alterations in the microenvironment of diabetic foot ulcers after stem cell application. Stem cells promoted M2 polarization of macrophages and secretion of anti-inflammatory cytokines. Stem cells increased the levels of PDGFA, HGF, NGF, and bFGF in the wound site, and decreased the levels of MMP-2, MMP-9, and proinflammatory cytokines. Stem cells promoted collagen deposition by acting on fibroblasts. Increased levels of local VEGF can recruit EPCs to the wound site and increase the levels of PDGF and FGF-2. EGF can promote the proliferation of keratinocytes, and TGF-β can regulate local immunity by increasing the levels of Treg cells. MSC, mesenchymal stem cell; PDGFA, platelet-derived growth factor A; HGF, hepatocyte growth factor; NGF, nerve growth factor; bFGF, basic fibroblast growth factor; MMP-2, matrix metaroprotease-2; MMP-9, matrix metaroprotease-9; FGF-2, fibroblast growth factor; EGF, epidermal growth factor; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α; Tregs, regulatory T cells; IFN-γ, interferon-γ; TGF-β, transforming growth factor-β.

stimulating hMSCs *via* a p38 MAPK-dependent mechanism (Wang et al., 2006). Cytokines and growth factors affect the activity of other cells (including immune cells) and are necessary for communication between various cells (e.g., fibroblasts, keratin-forming cells, immune cells, and endothelial cells) (Matthay et al., 2010). A study concluded that VEGF could

**TABLE 2 |** Documented efficacy and limitations of extended application of stem cell-based therapies and brief summary of major trials for each modality.

| Extended application of stem cell-based therapies | Efficacy | Limitations | Completed or ongoing trials | Interventions |
|---|---|---|---|---|
| Biomaterials | Enhance the repair efficiency and vitality of stem cells, improve the inflammatory microenvironment, avoid immune rejection, and improve the retention and survival rate of stem cells | — | Safety, tolerability and efficacy of CYP-006TK in adults with diabetic foot ulcers | Combination product: CYP-006TK |
| | | | Clinical study to evaluate efficacy and safety of ALLO-ASC-DFU in patients with diabetic wagner grade 2 foot ulcers | Biological: ALLO-ASC-DFU\| procedure: vehicle sheet |
| | | | Clinical Study of ALLO-ASC-SHEET in Subjects with Diabetic Wagner Grade II Foot Ulcers | Biological: ALLO-ASC-SHEET |
| | | | Treatment of Chronic Wounds in Diabetic Foot Syndrome with Allogeneic Adipose Derived Mesenchymal Stem Cells | Biological: application of allogeneic ADSC stem cells in fibrin gel\| Procedure: standard care in diabetic foot ulcer |
| | | | Clinical Study of ALLO-ASC-SHEET in Subjects with Diabetic Foot Ulcers | Biological: ALLO-ASC-DFU\| procedure: hydrogel sheet (vehicle control) |
| Culture medium and Exosomes | Modulation of signal transduction between target cells by small molecules | Increases the risk of tumorigenicity and exogenous contamination | — | |
| Gene therapy | Enhance cell performance and improve the survival rate of stem cells | — | Safety and Efficacy study of neovasculgen (Pl-VEGF165) gene therapy in patients with diabetic foot | Neovasculgen |
| Small molecule compounds and drug pretreatment | Improve the survival rate, proliferation, migration and paracrine ability of stem cells | Poor bioactivity and stability of small molecules or drugs | — | |
| Hypoxic preconditioning | Improve the tolerance of cells in the hypoxic environment | — | — | |

*Reference: https://ClinicalTrials.gov/.*

locally upregulate platelet-derived growth factor B (PDGF-B) and fibroblast growth factor-2 (FGF-2) in wounds (Galiano et al., 2004) and systematically mobilize and recruit bone marrow-derived cells (including endothelial progenitor cells (EPCs)) into the local microenvironment of wound site to improve wound healing and tissue remodeling (Galiano et al., 2004; Ishida et al., 2019).

Neovascularization, a critical step for the process of wound healing, was consistently depressed by hyperglycemic conditions at the wound sites, as revealed by endothelial cell marker CD31 (Ishida et al., 2019). MSCs can significantly improve diabetic foot ulcers by producing angiogenic factors such as VEGF. Compared with non-diabetic rats, the expression level of VEGF protein in diabetic rats was significantly reduced (Schratzberger et al., 2001). MicroRNA-205-5p is a direct regulator of VEGF protein translation and is expressed in hMSCs (Zhu et al., 2017). Zhu et al. (2019) found that microRNA-205-5p could specifically target 3′-UTR of VEGF mRNA to inhibit the translation of VEGF protein, thus inhibiting wound healing of diabetic foot (Zhu et al., 2017). MALAT1 is a competing endogenous RNA (ceRNA) competing with microRNA-205-5p. The expression of MALAT1 was downregulated in tissue biopsies of DFU patients (Jayasuriya et al., 2020). In the DF model established by immunodeficient NOD/SCID mice, the overexpression of MALAT1 in MSCs significantly downregulated the level of microRNA-205-5p, led to the up-regulation of VEGF production and improved the formation of endothelial cell tubes (Zhu et al., 2019). Deletion of microRNA-205-5p and overexpression of MALAT1 did not substantially change the mRNA level of VEGF but significantly enhanced the protein level of VEGF in MSCs and MALAT1 serves as a post-transcriptional activator to increase the expression of VEGF protein (Zhu et al., 2019). MALAT1 silencing can reduce the expression level of Collagen I and Collagen III in the skin of diabetic mice, thus reduce the deposition of collagen at the wound sites and delay wound healing (Liu et al., 2019). Collagen is secreted by fibroblasts (Vig et al., 2017). These studies indicate that MALAT1 promotes ulcer healing by promoting VEGF secretion or acting on fibroblasts to promote collagen secretion. The level of Nrf2 in the circulation of diabetic patients is lower (Sireesh et al., 2018). In diabetic foot ulcers, Nrf2 can upregulate the expression of MALAT1 through a positive feedback mechanism (Jayasuriya et al., 2020). Exosomes from adipose-derived stem cells (ADSCs)

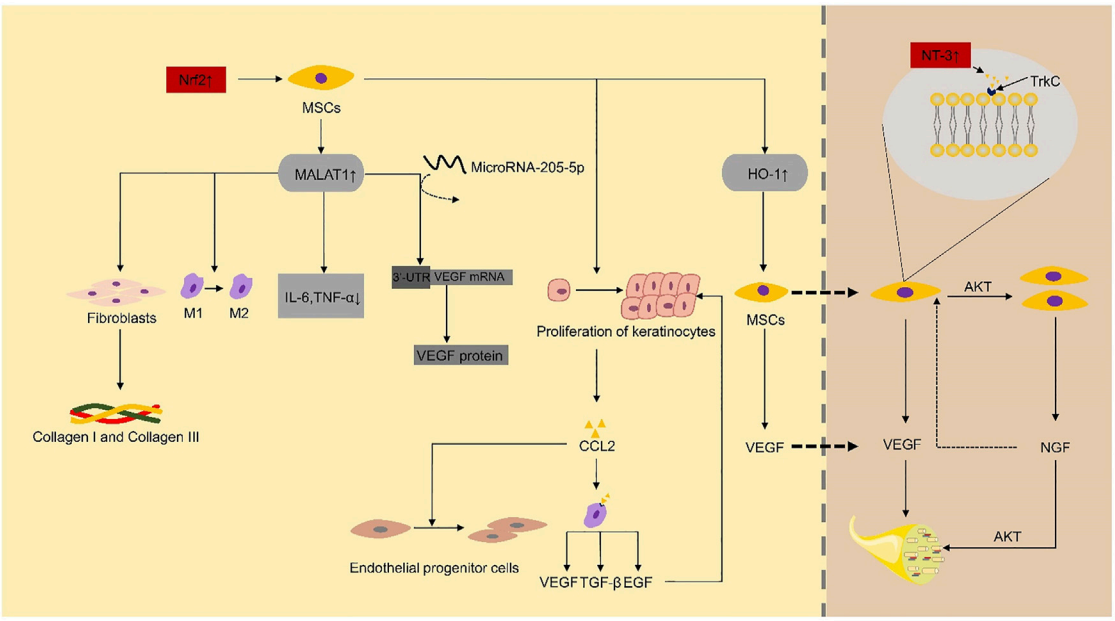


**FIGURE 2 |** Nrf2 and NT-3 play a critical role as key factors in the repair of diabetic foot ulcers by mesenchymal stem cells. IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; MSCs, mesenchymal stem cells; NGF, nerve growth factor; Trk, tropomyosin receptor kinase.

overexpressing Nrf2 improve angiogenesis and proliferation of EPCs, which accelerate cutaneous wound repair in DFUs (Li et al., 2018). These results support a crucial role for Nrf2 in modulating the effects of MSC-based therapies in the unfavorable *in vivo* microenvironment (**Figure 2**). Furthermore, increasing the local MALAT1 level of wounds can significantly improve wound inflammation and promote wound healing. For example, MALAT1-overexpressed MSCs can promote M2 macrophage polarization (Li et al., 2017) and decrease the expression of proinflammatory cytokines IL-6 and TNF-α (Jayasuriya et al., 2020).

Nrf2-dependent genes, such as heme oxygenase-1 (HO-1), play a vital role in the regulation of angiogenesis and the increased expression of angiogenic factors (Loboda et al., 2016). Hou et al. (2013) reported that the overexpression of HO-1 in BMSCs in STZ-induced diabetic mice could promote the proliferation of BMSCs and enhance the secretion of VEGF by BMSCs through the Akt signal pathway, which eventually promotes wound ulcers repair significantly.

MFG-E8 is expressed perivascularly and intravascularly, and it is essential for VEGF-induced AKT phosphorylation (Silvestre et al., 2005). VEGF and PDGF, as crucial growth factors for the induction of angiogenesis, require MFG-E8-mediated signaling (Silvestre et al., 2005; Uchiyama et al., 2014). A study confirmed that BM-MSCs could promote angiogenesis by overexpressing MFG-E8 (Uchiyama et al., 2017). Although this study also shows that MFG-E8 can promote M2 macrophage infiltration and inhibit proinflammatory cytokines, thus promoting diabetic wound healing, the underlying mechanism of MFG-E8 regulating inflammation is not clear.

PI3K/Akt signal pathway is essential for angiogenesis (Karar and Maity, 2011). Nerve growth factor (NGF), which binds to its specific receptor TrkA (Storkebaum and Carmeliet, 2011), can promote the proliferation of endothelial cells in blood vessels and stimulates MSC to produce VEGF (Shen et al., 2013) and nitric oxide synthase (NOS) through PI3K/Akt pathway to promote angiogenesis (Emanueli et al., 2002; Emanueli et al., 2003). MSCs expressed NT-3 specific receptor type 3 neurotrophin tyrosine kinase receptor (TrkC) (Storkebaum and Carmeliet, 2011). NT-3-activated hMSC can promote foot wound healing in diabetic mice. Shen et al. (2013) reported that NT-3 upregulates the proliferation of hMSC through the Akt pathway. NT-3 can also upregulate the secretion of VEGF and NGF proteins in MSCs, and these growth factors can enhance the activity of vascular endothelial cells and promote angiogenesis through Akt pathway (Shen et al., 2013).

The c-jun expression of MSCs decreased gradually in the process of subcultivation (Yue et al., 2020). At the diabetic wound sites, the expression of c-jun also decreased (Tombulturk et al., 2019). Yue et al. (2020) found that local subcutaneous injection of hUC-MSCs overexpressing c-jun at the diabetic wound sites accelerated angiogenesis and re-epithelialization by increasing the levels of PDGFA and HGF. PDGFs are the major mitogens for several cells types of mesenchymal origin, such as smooth muscle cells and fibroblasts (Fredriksson et al., 2004). Activator protein-1 (AP-1) is one of the main downstream effectors of MAPKs (Kajanne et al., 2007), and c-Jun, the main component of the AP-1, has crucial functions in cellular proliferation (Davies et al., 2013). The c-jun expression of MSCs plays a vital role in promoting diabetic

foot wound repair. MSCs overexpressing c-jun may promote wound repair by downregulating the expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9). MMP-2 and MMP-9 are overexpressed in diabetic chronic ulcers (Wysocki et al., 1993). AP-1 has shown to be the main regulator of MMP-2 and MMP-9 transcription under various conditions, as well as in diabetic wounds (Tombulturk et al., 2019). Although the local application of bone marrow MSCs resulted in increased levels of MMP-2 and MMP-9 expression in diabetic wounds compared to the control group, they showed a decreasing trend (Lv et al., 2017). In the process of wound healing, MMP-2 plays an essential role in prolonged matrix remodeling and angiogenesis (Tombulturk et al., 2019), and MMP-9 is thought to be involved in granulation tissue remodeling and keratinocyte migration (Salo et al., 1994). In addition, there has been evidence that down-regulation of MMP-2 and MMP-9 by c-jun can promote wound healing in diabetic mice (Tombulturk et al., 2019), and the overexpression of MMP-2 and MMP-9 can impair wound healing through the degradation of skin extracellular matrix (ECM) (Krishnan et al., 2018).

## Repairing Diabetic Foot Through Anti-Inflammatory and Immunomodulation

Long-term inflammation can lead to a hypoxic environment and abnormal produc tion of angiogenic signals. The anti-inflammatory and immunomodulatory effects of implanted stem cells have been suggested as a potential mechanism for the repairing effects of stem cell-based therapies. Inflammation has a regulatory effect on immune function. Low-grade inflammation is beneficial to wound repair, which helps to remove invading pathogens, promotes the repair of damaged tissues, and maintains homeostasis. Continuous and excessive inflammation leads to delayed or nonhealing of diabetic foot wound ulcers (Eming et al., 2007). Excessive inflammation and immune suppression often coexist. The excessive immune response can cause severe systemic inflammation or allergic diseases, while an extremely low immune response can easily induce severe or frequent infection. Therefore, the balance between proinflammatory and anti-inflammatory during varying repair stages is essential to achieving tissue homeostasis following tissue injury. Loretelli et al. (2020) found that the topical application of embryonic stem cell extracts (EXTs) in diabetic foot mice can promote wound healing of diabetic foot, which is accompanied by a decrease in $CD45^+$ inflammatory cells and interferon-γ (IFN-γ), while an increase in regulatory T cells (Tregs), proliferating Ki-67+ cells, and the endothelial cell marker CD31. This finding indicates that the promotion of diabetic wound healing by ESCs is related to immune regulation. It is well known that stem cells have anti-inflammatory and immunomodulatory properties, so it is of great significance to explore the relationship between the immunomodulatory properties of ESCs and wound healing.

Delayed increase of macrophages and prolonged inflammatory stage can be found in diabetic mice. The local proinflammatory response in the MSC group was significantly reduced comparing with the control group, and the expression of CD45 was inhibited, proving that MSCs play an anti-inflammatory effect in repairing diabetic foot ulcers (Kuo et al., 2011; Yang et al., 2020a). Furthermore, the abnormal expression of chemokines may be the underlying cause of delayed wound healing in diabetic mice (Wetzler et al., 2000). A study has shown that the concentration of CCL2 in diabetic wounds is reduced, and an enhanced expression of CCL2 can reverse the impaired skin wound healing in diabetic mice by mediating neovascularization and normalization of collagen accumulation (Ishida et al., 2019). Macrophages were the main source of VEGF and TGF-β in the wound, and diabetes can delay the increase of macrophages at the injured site. More than a few studies have demonstrated MSCs can enhance the levels of VEGF and TGF-β at the diabetic wound sites (Kuo et al., 2011; Wan et al., 2013; Yang et al., 2020b). Yang et al. (2020a) demonstrated that the expression of CCL2 was considerably upregulated after topical application of MSCs, and angiogenesis was markedly improved during wound healing. Macrophages express CCL2 receptors (CCR2). Therefore, it can be speculated that CCL2 plays an important role in promoting wound repair by reversing the reduced macrophage infiltration and enhance the levels of VEGF and TGF-β. TGF-β can directly promote the proliferation of Treg cells and regulate macrophages (Batlle and Massagué, 2019). Nrf2 directly regulates the secretion of CCL2 by epidermal keratinocytes (Villarreal-Ponce et al., 2020). Kato et al. (2014) have shown that BM-MSCs can improve wound healing of plantar skin ulcers in diabetic rats by enhancing the function of keratinocytes. Therefore, we can speculate that MSCs may increase the secretion of CCL2 by promoting the functional recovery of keratinocytes, which can reverse the decrease of macrophage infiltration and ultimately promote ulcer repair. CCL2 can also regulate the production of EGF in macrophages at the site of injury, and EGF can induce the proliferation of keratinocytes (Villarreal-Ponce et al., 2020).

Hyperglycemic conditions have also been shown to reduce the number of EPCs and impair their function and recruitment to the injured site (Tepper et al., 2002; Ishida et al., 2019). The increased levels of CCL2 expression can further enhance the accumulation of EPCs in the wound site of diabetic mice and ultimately accelerate the formation of new blood vessels (Ishida et al., 2019).

Diabetic chronic nonhealing wounds are characterized by the increase of proinflammatory cytokines, including IL-1, IFN-γ, TNF- α, and IL-6, which are mainly produced by activated macrophages and play an important role in the regulation of immune cells (Zubair and Ahmad, 2019). It is suggested that the decrease and activation of macrophages may result in the damage of diabetic wound healing (Maruyama et al., 2007). Topical application of MSCs may modulate the inflammatory response by secreting the anti-inflammatory cytokine IL-10. Wang et al. (2016) found that combined treatment with PMSCs and IL-10 antibodies significantly delayed diabetic wound healing compared to treatment with PMSCs alone. Furthermore, topical application of PMSCs significantly reduced local levels of the proinflammatory cytokines IL-1, TNF-α, and IL-6. The wound inflammation reaction time was significantly prolonged in db/db mice, and the strong increase of proinflammatory cytokines (e.g., IL-1β and TNF-α) could still be detected in the

later stage of the wound repair (Wetzler et al., 2000). The presence of PMSCs was found to strongly inhibit LPS-induced activation of NF-κB in dermal fibroblasts. Since NF-κB plays a central role in regulating the transcription of the proinflammatory cytokines IL-1, TNF-α, and IL-6, it is hypothesized that PMSCs may regulate the inflammatory response in diabetic foot wound healing by targeting NF-κB (Wang et al., 2016). In addition, the two proinflammatory cytokines, IL-1β and IL-6, were significantly reduced in wounds of db/db mice treated with the MSC combination regimen (Yang et al., 2020a), supporting its immunomodulatory function. In conclusion, these findings indicate that MSCs promote cutaneous wound healing, at least in part, by inhibiting the secretion of proinflammatory cytokines and by increasing the production of anti-inflammatory cytokines at local wound sites.

Diabetic wounds are characterized by disturbance of macrophage phagocytosis, delayed macrophage infiltration, and inflammatory response disorder. Proinflammatory cytokines (IL-6, TNF-α, IFN-γ) are known to induce M1 macrophages (Gu et al., 2013). M2 macrophage is characterized by the production of TGF-β and IL-10 (Zhang S et al., 2020), which can be induced by anti-inflammatory cytokines, including IL-4, IL-10, and TGF-β (Gu et al., 2013). M2 macrophages have been shown to promote angiogenesis, improve nerve damage, and inhibit inflammation. The microenvironment of wound inflammation determines the phenotype of macrophages. Topical application of stem cells to wounds can promote the conversion of macrophages to an anti-inflammatory phenotype (M2) (Yang et al., 2020a). Depletion of Treg leads to impaired healing, characterized by impaired vascular maturation and delayed re-epithelialization (Haertel et al., 2018). A study has shown that intramuscular injection of hUC-MSCs into patients with diabetic foot can significantly increase the ratios of Treg/Th17 and Treg/Th1 cells (Li et al., 2013). And then, Treg cells induce M2 macrophage differentiation through TGF-β and IL-10 pathways (Liu et al., 2011). Furthermore, M2 macrophages can improve the local inflammatory microenvironment of wounds and thus promote angiogenesis. It is reported that umbilical cord-matrix stem cells (UCMSCs) polarized macrophages can promote high glucose-induced functional recovery of human umbilical vein endothelial cells (Zhang S et al., 2020).

## Repairing Diabetic Foot by Improving Nerve Blood Supply and Reducing Chronic Nerve Inflam Mation

Peripheral neuropathy and microangiopathy of the diabetic foot often develop and aggravate at the same time. Therefore, the repair of nerves may also be one of the possible mechanisms for MSCs to repair diabetic foot ulcers. In fact, the number of blood vessels and nerve blood flow reduced significantly in streptozotocin (STZ)-induced diabetic rats, and intramuscular gene transfer of naked DNA encoding angiogenic growth factors VEGF-1 or VEGF-2 actually led to the resolution of diabetic neuropathy (Schratzberger et al., 2001). Since VEGF improves the blood vessels and blood flow in the nerves in the model, the effect of VEGF in promoting vascular perfusion seems to be related to the improvement of neuropathic diabetic foot. Notably, NGF and NT-3 can reverse diabetic neuropathy (Casellini and Vinik, 2007) (**Figure 2**). Therefore, we can speculate that NGF and NT-3 can improve neuropathic diabetic foot by acting on VEGF directly or indirectly. Human UC-MSCs improved the neurodegeneration of the femoral nerve in DFU rats and showed an increase in serum NGF and angiogenesis, which further confirmed this view (Xia et al., 2015). In addition, ADSCs can reverse the degeneration of large nerve fibers, promote neuronal axonal regeneration, and improve peripheral nerve perfusion (De Gregorio et al., 2020).

Chronic inflammation of the peripheral nerve directly leads to chronic pain and neurodegeneration (Vincent et al., 2011). De Gregorio et al. (2020) demonstrated that adipose-derived stem cells (ADSCs) can reduce chronic inflammation of the sciatic nerve by upregulating anti-inflammatory cytokines IL-1β and TNF-α. Dash et al. (2009), Wang et al. (2019) reported that patients with DFUs had a significant improvement in painless walking distance after using BM-MSCs.

## Other Mechanisms: Transdifferentiation Into Epithelial Cells, Collagen Deposition Etc

In addition to promoting angiogenesis through paracrine, hUC-MSCs can directly transdifferentiate into epithelial cells and endothelial cells, which play a role in promoting the repair of diabetic foot ulcers (Shi et al., 2020). $CD31^{+}$ cells were presumed to identify transdifferentiated endothelial cells. Consistent with the study that ADSCs can also differentiate into endothelial cells and promote angiogenesis (An et al., 2020), it is suggested that hMSCs have similar characteristics, although they may be through different signal pathways, for example, MAPK/ERK signaling pathway mediates VEGF-induced differentiation of BM-MSCs to endothelial cells (Xu et al., 2008), and PI3K signaling pathway mediates VEGF-induced differentiation of ADSCs to endothelial cells (Cao et al., 2005). However, it is not clear through which signal pathway mediates the differentiation of hUC-MSCs to endothelial cells.

MSCs can improve collagen synthesis and promote collagen deposition in diabetic foot wounds (Wang et al., 2016; Shi et al., 2020). Collagen is mainly expressed and distributed in the dermis of the skin, secreted by fibroblasts, and is closely related to the repair of damaged skin (Vig et al., 2017). Fibroblasts isolated from diabetic foot wounds show lower proliferative potential and reduced production of growth factors (Robson, 2003). ADSCs from diabetic foot wounds can be directly transformed into fibroblasts, which significantly promote the expression of collagen I and collagen III (An et al., 2020). Jung et al. (2018) healthy fibroblasts and human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) were used separately to co-culture with diabetic fibroblasts to study their effects on cell proliferation and collagen synthesis. The results showed that hUBC-MSCs themselves did not secrete more ECM components than fibroblasts, but hUBC-MSCs stimulated the synthesis of ECM (including collagen) in diabetic fibroblasts much more than

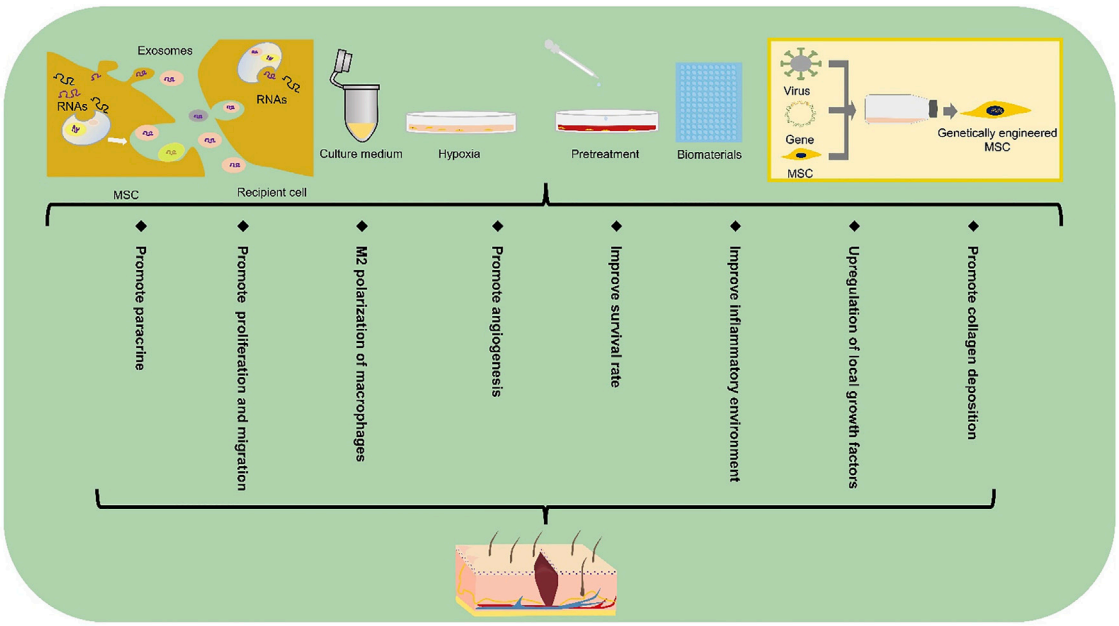


**FIGURE 3 |** Extended application of stem cell-based therapy in DFUs. Extended applications for stem cell repair of the diabetic foot include exosomes, gene therapy, biomaterials, culture media, pretreatment and hypoxic preconditioning. MSC, mesenchymal stem cell.

in fibroblasts. Therefore, MSCs may promote granulation tissue formation in diabetic foot wounds by promoting fibroblast proliferation and functional recovery, thereby prompting fibroblasts to secrete more ECM and growth factors, and ultimately promoting wound healing.

In fact, the process of stem cell repair of diabetic foot is not a single factor acting independently; various mechanisms are interconnected and interact with each other to ultimately promote damage repair. For example, the M2 polarization of macrophages accelerates peripheral nerve repair (Mokarram et al., 2012; Pacific et al., 2020).

## EXTENDED APPLICATION OF STEM CELL-BASED THERAPY IN DFUS

The decrease in stem cell survival rate caused by late glycosylation end products in a hyperglycemic environment severely diminishes the efficiency of stem cell repair (Wang et al., 2015). If the repair efficiency is improved by increasing the number of locally applied stem cells, it may increase tumorigenicity and other adverse consequences (Mathew et al., 2016). Moreover, after multiple passages of cells *in vitro*, the potential of multi-directional differentiation and paracrine ability may also decrease. Therefore, there is an urgent need for a method to improve the survival rate and paracrine ability of stem cells. Researchers have begun to develop stem cell-based derived wound repair therapies, which aims to improve the vitality and repair efficiency of stem cells at the wound site. Here, we talk about the extended applications of stem cells (**Figure 3**).

### Biomaterials

Typical chronic nonhealing wounds show reduced levels of growth factors. Therefore, the wound microenvironment is particularly crucial in wound healing, especially under certain health conditions. Stem cells play a crucial role in regulating the local microenvironment of diabetic foot ulcers. More importantly, these roles have been observed in clinically chronic injury-related diseases, including DFUs (Li et al., 2013). However, the hyperglycemic inflammatory microenvironment may reduce the biological activity of stem cells as well as accelerate the degradation of their secreted cytokines and growth factors. Therefore, a more effective approach to suppress the negative effects of the microenvironment on stem cells appears essential. In recent years, considerable efforts have been contributed to better enhance the repair efficiency and vitality of stem cells and improve the inflammatory microenvironment by using biomaterials as the carrier of stem cell application. Hydrogel has been shown to alleviate the inflammatory environment of the wound, thus promoting the secretion of growth factors from MSCs (Bai et al., 2020) and improving the innervation of diabetic foot (da Silva et al., 2017). Furthermore, Zhao et al. (2020a) managed to improve wound healing by applying hyaluronic acid hydrogel combined with UC-MSCs and endothelial colony-forming cells (ECFCs), which had a significantly positive effect on human refractory diabetic chronic wounds.

ECM-based biomaterials (such as cell sheets and decellularized cell sheet grafts) greatly facilitate stem cell implantation due to their low immunogenicity and resistance to the body's adverse microenvironment (Capella-Monsonís et al., 2020). Tissue-engineered cell sheets are composed of ECM and cells. It uses

temperature-sensitive culture systems or enzyme-sensitive culture systems to naturally desorb cells from the substrate surface and eventually obtain a cell sheet layer structure containing ECM (Zhou et al., 2020). The cell sheet technology can avoid immune rejection caused by exogenous foreign substances such as scaffold materials and maintain the three-dimensional structure of the ECM (Guo et al., 2021). Stem cells, as potential cells with strong regenerative capacity, are the best choice for tissue engineering cell sheets to play a role in damage repair. Adipose stem cell sheets have been shown to accelerate diabetic ulcer healing by upregulating local growth factors (Kato et al., 2015). Although there are no current experiments to validate the survival rate of MSCs at the diabetic foot ulcer sites after cell sheet transplantation, experiments by Guo et al. (2021) demonstrated higher retention and survival rate of stem cell sheets transplanted into the infarcted myocardium than cell suspensions. This provides new hope for improving the survival rate of stem cells from diabetic wound sites. Furthermore, cell sheet-based 3D printing technology makes it possible to form specific shapes to match the structure of the administered tissue or organ while maintaining the structural integrity of the ECM (Bakirci et al., 2017).

Other biomaterials such as peptide-modified biomaterials (Gallagher et al., 2020), cytokine-modified biomaterials (García et al., 2019), injectable biomaterials (Han et al., 2020), and programmable microcapsules (Mao et al., 2019) have also improved the survival rate of cells at the transplantation site to varying degrees, which greatly facilitates MSCs to fully exploit their role in repairing the damage.

## Culture Medium and Exosomes

When stem cells are applied locally to a wound, some of the cells may die in the short term due to hypoxia caused by the local high glycemic environment. However, most experiments have demonstrated that stem cells reach the level of healing injury, can these dead stem cells release a series of bioactive substances to repair the damage after lysis? Moreover, if the bioactive substances released by the dead cells can repair the damage, can there be other alternative methods to replace the direct application of stem cells? It is worth thinking about. Some of the bioactive substances produced by MSCs through paracrine secretion exist in the culture medium used in the culture process of MSC *in vitro*, and these bioactive substances play a crucial role in promoting damage repair. Studies have been conducted to demonstrate that the application of MSC culture medium for the treatment of diabetic foot can achieve the same results as the application of MSCs (Zhang S. et al., 2020). Therefore, the application of MSC conditioned media also brings new hope for patients with diabetic foot ulcers.

Although the application of stem cells has entered the clinical stage, their safety issues still need extensive attention. The weak immunogenicity of stem cells has led to the widespread use of stem cells from different sources in injury repair. However, because of their weak immunogenicity, the application of allogeneic stem cells greatly evades the surveillance of the body's own immune system and therefore increases the risk of tumorigenicity. In addition, MSCs are highly susceptible to exogenous contamination during their *in vitro* preparation due to inconsistent preparation specifications, which can be extremely harmful to the human body. Most of the therapeutic effects of MSCs are mediated through paracrine secretion, with the most attention being paid to the release of small molecules, exosomes, by stem cells through exocytosis. Exosomes contain a variety of molecules, including proteins, mRNAs, and microRNAs (miRNAs), which can act as novel intercellular communication tools to regulate signaling between target cells. For example, exosome-regulated fibroblasts receive exosome-targeted signals in the form of internalized exosomes and actively regulate collagen production including promotion of type I and type III collagen production during the pre-wound healing phase and inhibition of collagen formation during the late wound healing phase to reduce scar formation (Dalirfardouei et al., 2019). Currently, MSC exosomes have shown promising results in diabetic wounds. For example, exosomes released from menstrual blood-derived mesenchymal stem cells (MenSCs) can improve diabetic foot wound healing by promoting macrophage M2 polarization and angiogenesis (Dalirfardouei et al., 2019). Huang et al. (2021) found that MSC-derived exosome miRNA-21-5p could promote angiogenesis in diabetic ulcers. Even more innovative, researchers have used UCMSCs-derived exosomes combined with hydrogels to promote diabetic foot wound repair (Yang et al., 2020b).

## Gene Therapy

Gene therapy aims to achieve the goal of targeted treatment of diseases by enhancing cell performance, continuously producing functional proteins, and inducing the expression of functional genes in tissues (Shomali et al., 2020). For example, PDGF-B-transfected MSCs can promote traumatic collagen deposition and angiogenesis (Li et al., 2007). A variety of exogenous target genes can be integrated into MSCs genomic DNA and expressed for a long period of time, which is why MSCs have been successfully used as an ideal target cell for the treatment of diseases such as hematological disorders, lung injury, and bone defects. In addition, transfected MSCs have been shown to improve the low survival rate of transplanted cells in diabetic wound sites (Khalid et al., 2019). Researchers have already transfected BM-MSCs with adenovirus-vectored VEGF genes, which significantly promoted the repair of diabetic foot wounds in rats (Cai et al., 2014). Compared with untransfected MSCs, IL-7-transfected BM-MSCs were able to enhance cell-cell connections in diabetic foot trauma as well as promote angiogenesis through induction of angiogenic genes (Khalid et al., 2019). Because of the ability to stably express target genes transduced into cells, exploring specific target genes to enhance the efficacy of MSCs in repairing diabetic foot injury offers a promising future for regenerative medicine.

## Small Molecule Compounds and Drug Pretreatment

The current pretreatment methods mainly include drug pretreatment and small molecular compound pretreatment. As pretreatment can effectively improve the survival rate and

paracrine ability of implanted stem cells in hyperglycemic microenvironment, it has been concerned by more and more scientific researchers. The researchers found that UCMSCs pretreated with small molecular compound 3,3′-diindolylmethane could enhance their proliferation and paracrine ability (Shi et al., 2017). In addition, pretreatment with the drug salidroside was able to increase the survival rate of MSCs and improve the migration ability of MSCs in a hyperglycemic microenvironment (Ariyanti et al., 2019). These studies support the positive effects of small molecules or drug pretreatment on stem cells. However, the bioactivity and stability of these small molecules or drugs *in vitro* and *in vivo* are important factors to be considered before applying them in pretreatment, and not all substances with proven *in vivo* or *in vitro* effects such as antioxidant and anti-apoptotic effects can be applied to enhance the repair efficiency of MSCs. For example, curcumin-pretreated MSCs have been shown to be able to withstand oxidative stress (Liu et al., 2015). However, due to the structural instability of curcumin, some scholars have questioned whether it really has some therapeutic efficacy *in vivo* (Nelson et al., 2017).

### Hypoxic Preconditioning

Hypoxic preconditioning can improve the survival rate and wound repair performance of MSC in ischemic and hypoxic environment, mainly through up-regulation of genes related to cell growth, metabolism and stress response pathway (Peck et al., 2019), up-regulation of epithelial regeneration-related genes and angiogenesis-related genes (Zhang X. R. et al., 2020), and restoration of paracrine function damaged in high glucose environment (Xu et al., 2020). The results of hypoxic preconditioning of MSCs by Peck et al. (2019) demonstrated the positive effects of preconditioned MSCs in avascular tissues such as intervertebral discs and cartilage. DFUs also face similar hypoxia as in the intervertebral discs or cartilage due to poor angiogenesis in the acquired wound site. Beegle et al. (2015) found that hypoxia-preconditioned MSCs injected intramuscularly into mice showed reduced glucose consumption and high survival rate. The hypoxic environment is simulated *in vitro* by increasing the $CO_2$ concentration (5%) or decreasing the oxygen concentration (1–2%) (Meng et al., 2018; Wang et al., 2018; Zhao et al., 2020b). Hypoxia inducible factor-1α (HIF-1α) is the main regulator involved in hypoxic preconditioning (Peck et al., 2019). HIF-1α expression tended to increase in stem cells as oxygen concentration decreased (Zhao et al., 2020b). Under hypoxic preconditioning conditions, lincRNA-p21 enhanced the repair efficiency of MSCs through the HIF-1α signaling pathway (Meng et al., 2018). HIF-1α positively regulates the expression of its downstream signaling genes such as VEGF, SDF-1a, FGF2, CXCL12, and GRP78 under hypoxic conditions (Lee et al., 2017; Zhao et al., 2020b; Xu et al., 2020). The expression and activity of HIF are suppressed in the hyperglycemic environment of diabetes (Catrina and Zheng, 2021), therefore, hypoxic preconditioning of MSCs appears to be essential in diabetic foot ulcers as a means of enhancing the expression of HIF, the upstream factors regulating damage-repair-related genes. After hypoxia treatment, the expression of cMET in MSCs increased and showed hyperphosphorylation in the early stage under the stimulation of HGF, and even in the later stage, cMET phosphorylation levels were comparable to those in normoxic cultured MSCs (Lee et al., 2017). In addition, hypoxic preconditioning has been demonstrated to improve the tolerance of cells in the corresponding hypoxic environment (Wang et al., 2018).

## CONCLUDING REMARKS

Stem cell-based therapies are promising in the field of regenerative medicine, and their mechanisms include promoting angiogenesis, ameliorating neuroischemia and inflammation, and promoting collagen deposition. However, little is known about their specific molecular mechanisms and biological properties. Although the application of stem cells has entered the clinical stage (**Table 2**), the discussion about their safety remains an inescapable part of the research. MSCs promote wound healing in a dose-dependent manner (O'Loughlin et al., 2013), but at the same time, promote tumor growth in a dose-dependent manner (Djouad et al., 2003). Currently, there are no clear standards regarding the dose of MSC application. Therefore, although stem cells show a bright future in regenerative medicine, there remain numerous challenges for researchers to overcome. In order to maximize the performance of stem cells in repairing skin damage, it is necessary to compare the efficacy and safety of different applications such as local application and systemic injection. Presently, the main ways of applying MSCs in wound sites are local injection, intramuscular injection, intravenous injection, arterial injection, and stent implantation. Systemic infusion requires a larger volume of stem cells to ensure that a sufficient number of stem cells are returned to the target tissue. Although stem cells can be isolated from many tissues, such as bone marrow, umbilical cord, fat, the number of isolated and purified stem cells is not considerable. The homing ability of stem cells after multiple passages was significantly weaker than that of freshly isolated stem cells (Rombouts and Ploemacher, 2003). This leads to an irreconcilable contradiction between the quantity and efficiency of stem cells. In addition, stem cells are blocked in the lungs after intravenous injection, which affects stem cells homing to inflammation or wound sites (Becker and Riet, 2016). Therefore, there remain many issues to be solved in the clinical application of stem cells.

## AUTHOR CONTRIBUTIONS

QY and G-HQ: Conception and design, collection and/or assembly of data, data analysis and interpretation, visualization, manuscript writing and final approval of the manuscript, these authors contributed equally to this work; LY and MW: collection and revision of data; HS and T-LM: conception and design, financial support, administrative support, provision of study material, supervision, collection and/or assembly of data, data analysis and interpretation, visualization, manuscript writing and final approval of the

manuscript. All authors reviewed the manuscript. All authors All authors have read and agreed to the published version of the manuscript.

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## REFERENCES

American Diabetes Association (2016). 9. Microvascular Complications and Foot Care. *Diabetes Care* 39 (Suppl. 1), S72–S80. doi:10.2337/dc16-S012

An, R., Zhang, Y., Qiao, Y., Song, L., Wang, H., and Dong, X. (2020). Adipose Stem Cells Isolated from Diabetic Mice Improve Cutaneous Wound Healing in Streptozotocin-Induced Diabetic Mice. *Stem Cel Res. Ther.* 11, 120. doi:10.1186/s13287-020-01621-x

Ariyanti, A. D., Zhang, J., Marcelina, O., Nugrahaningrum, D. A., Wang, G., Kasim, V., et al. (2019). Salidroside-Pretreated Mesenchymal Stem Cells Enhance Diabetic Wound Healing by Promoting Paracrine Function and Survival of Mesenchymal Stem Cells under Hyperglycemia. *Stem Cell Transl. Med.* 8, 404–414. doi:10.1002/sctm.18-0143

Armstrong, D. G., Boulton, A. J. M., and Bus, S. A. (2017). Diabetic Foot Ulcers and Their Recurrence. *N. Engl. J. Med.* 376, 2367–2375. doi:10.1056/nejmra1615439

Bai, H., Kyu-Cheol, N., Wang, Z., Cui, Y., Liu, H., Liu, H., et al. (2020). Regulation of Inflammatory Microenvironment Using a Self-Healing Hydrogel Loaded with BM-MSCs for Advanced Wound Healing in Rat Diabetic Foot Ulcers. *J. Tissue Eng.* 11, 2041731420947242. doi:10.1177/2041731420947242

Bakirci, E., Toprakhisar, B., Zeybek, M. C., Ince, G. O., and Koc, B. (2017). Cell Sheet Based Bioink for 3D Bioprinting Applications. *Biofabrication* 9, 024105. doi:10.1088/1758-5090/aa764f

Barwick, A. L., Tessier, J. W., Janse de Jonge, X., Ivers, J. R., and Chuter, V. H. (2016). Peripheral Sensory Neuropathy Is Associated with Altered Postocclusive Reactive Hyperemia in the Diabetic Foot. *BMJ Open Diab. Res. Care* 4, e000235. doi:10.1136/bmjdrc-2016-000235

Batlle, E., and Massagué, J. (2019). Transforming Growth Factor-β Signaling in Immunity and Cancer. *Immunity* 50, 924–940. doi:10.1016/j.immuni.2019.03.024

Becker, A. D., and Riet, I. V. (2016). Homing and Migration of Mesenchymal Stromal Cells: How to Improve the Efficacy of Cell Therapy? *World J. Stem Cell* 8, 73–87. doi:10.4252/wjsc.v8.i3.73

Beegle, J., Lakatos, K., Kalomoiris, S., Stewart, H., Isseroff, R. R., Nolta, J. A., et al. (2015). Hypoxic Preconditioning of Mesenchymal Stromal Cells Induces Metabolic Changes, Enhances Survival, and Promotes Cell Retention *In Vivo*. *Stem Cells* 33, 1818–1828. doi:10.1002/stem.1976

Bogliotti, Y. S., Wu, J., Vilarino, M., Okamura, D., Soto, D. A., Zhong, C., et al. (2018). Efficient Derivation of Stable Primed Pluripotent Embryonic Stem Cells from Bovine Blastocysts. *Proc. Natl. Acad. Sci. USA* 115, 2090–2095. doi:10.1073/pnas.1716161115

Boulton, A. J. M. (2010). The Diabetic Foot. *Medicine* 38, 644–648. doi:10.1016/j.mpmed.2010.08.011

Bus, S. A. (2012). Priorities in Offloading the Diabetic Foot. *Diabetes Metab. Res. Rev.* 28, 54–59. doi:10.1002/dmrr.2240

Cai, Q., Wan, J. B., Liang, W. J., and Liu, Y. (2014). Transplantation of Bone Marrow Mesenchymal Stem Cells Transfected by Vascular Endothelial Growth Factor Gene Promotes Foot Wound Healing in Diabetic Rats. *J. Diabetes Res.* 18, 5988–5992.

Cao, Y., Sun, Z., Liao, L., Meng, Y., Han, Q., and Zhao, R. C. (2005). Human Adipose Tissue-Derived Stem Cells Differentiate into Endothelial Cells *In Vitro* and Improve Postnatal Neovascularization *In Vivo*. *Biochem. Biophysical Res. Commun.* 332, 370–379. doi:10.1016/j.bbrc.2005.04.135

Capella-Monsonís, H., De Pieri, A., Peixoto, R., Korntner, S., and Zeugolis, D. I. (2020). Extracellular Matrix-Based Biomaterials as Adipose-Derived Stem Cell Delivery Vehicles in Wound Healing: a Comparative Study between a Collagen Scaffold and Two Xenografts. *Stem Cel Res. Ther.* 11, 510. doi:10.1186/s13287-020-02021-x

Casellini, C. M., and Vinik, A. I. (2007). Clinical Manifestations and Current Treatment Options for Diabetic Neuropathies. *Endocr. Pract.* 13, 550–566. doi:10.4158/ep.13.5.550

Catrina, S.-B., and Zheng, X. (2021). Hypoxia and Hypoxia-Inducible Factors in Diabetes and its Complications. *Diabetologia* 64, 709–716. doi:10.1007/s00125-021-05380-z

Cavanagh, P. R., Lipsky, B. A., Bradbury, A. W., and Botek, G. (2005). Treatment for Diabetic Foot Ulcers. *The Lancet* 366, 1725–1735. doi:10.1016/s0140-6736(05)67699-4

Clinicaltrials.gov (2021). *40 Clinical Trials Have Been Found on a Search for "Diabetic Foot Ulcers, Stem Cell"*. updated on February 5,2021.

da Silva, L. P., Santos, T. C., Rodrigues, D. B., Pirraco, R. P., Cerqueira, M. T., Reis, R. L., et al. (2017). Stem Cell-Containing Hyaluronic Acid-Based Spongy Hydrogels for Integrated Diabetic Wound Healing. *J. Invest. Dermatol.* 137, 1541–1551. doi:10.1016/j.jid.2017.02.976

Dalirfardouei, R., Jamialahmadi, K., Jafarian, A. H., and Mahdipour, E. (2019). Promising Effects of Exosomes Isolated from Menstrual Blood-derived Mesenchymal Stem Cell on Wound-healing Process in Diabetic Mouse Model. *J. Tissue Eng. Regen. Med.* 13, 555–568. doi:10.1002/term.2799

Dash, N. R., Dash, S. N., Routray, P., Mohapatra, S., and Mohapatra, P. C. (2009). Targeting Nonhealing Ulcers of Lower Extremity in Human through Autologous Bone Marrow-Derived Mesenchymal Stem Cells. *Rejuvenation Res.* 12, 359–366. doi:10.1089/rej.2009.0872

Davies, C. C., Chakraborty, A., Diefenbacher, M. E., Skehel, M., and Behrens, A. (2013). Arginine Methylation of the C-Jun Coactivator RACO-1 Is Required for C-Jun/AP-1 Activation. *Embo J.* 32, 1556–1567. doi:10.1038/emboj.2013.98

De Gregorio, C., Contador, D., Díaz, D., Cárcamo, C., Santapau, D., Lobos-Gonzalez, L., et al. (2020). Human Adipose-Derived Mesenchymal Stem Cell-Conditioned Medium Ameliorates Polyneuropathy and Foot Ulceration in Diabetic BKS Db/db Mice. *Stem Cel Res. Ther.* 11, 168. doi:10.1186/s13287-020-01680-0

Ding, D.-C., Shyu, W.-C., and Lin, S.-Z. (2011). Mesenchymal Stem Cells. *Cel Transpl.* 20, 5–14. doi:10.3727/096368910x

Djouad, F., Plence, P., Bony, C., Tropel, P., Apparailly, F., Sany, J., et al. (2003). Immunosuppressive Effect of Mesenchymal Stem Cells Favors Tumor Growth in Allogeneic Animals. *Blood* 102, 3837–3844. doi:10.1182/blood-2003-04-1193

Emanueli, C., Salis, M. B., Pinna, A., Graiani, G., Manni, L., and Madeddu, P. (2002). Nerve Growth Factor Promotes Angiogenesis and Arteriogenesis in Ischemic Hindlimbs. *Circulation* 106, 2257–2262. doi:10.1161/01.cir.0000033971.56802.c5

Emanueli, C., Schratzberger, P., Kirchmair, R., and Madeddu, P. (2003). Paracrine Control of Vascularization and Neurogenesis by Neurotrophins. *Br. J. Pharmacol.* 140, 614–619. doi:10.1038/sj.bjp.0705458

Eming, S. A., Krieg, T., and Davidson, J. M. (2007). Inflammation in Wound Repair: Molecular and Cellular Mechanisms. *J. Invest. Dermatol.* 127, 514–525. doi:10.1038/sj.jid.5700701

Forbes, J. M., and Cooper, M. E. (2013). Mechanisms of Diabetic Complications. *Physiol. Rev.* 93, 137–188. doi:10.1152/physrev.00045.2011

Fredriksson, L., Li, H., and Eriksson, U. (2004). The PDGF Family: Four Gene Products Form Five Dimeric Isoforms. *Cytokine Growth Factor. Rev.* 15, 197–204. doi:10.1016/j.cytogfr.2004.03.007

Galiano, R. D., Tepper, O. M., Pelo, C. R., Bhatt, K. A., Callaghan, M., Bastidas, N., et al. (2004). Topical Vascular Endothelial Growth Factor Accelerates Diabetic Wound Healing through Increased Angiogenesis and by Mobilizing and Recruiting Bone Marrow-Derived Cells. *Am. J. Pathol.* 164, 1935–1947. doi:10.1016/s0002-9440(10)63754-6

Gallagher, L. B., Dolan, E. B., O'Sullivan, J., Levey, R., Cavanagh, B. L., Kovarova, L., et al. (2020). Pre-culture of Mesenchymal Stem Cells within RGD-Modified Hyaluronic Acid Hydrogel Improves Their Resilience to Ischaemic Conditions. *Acta Biomater.* 107, 78–90. doi:10.1016/j.actbio.2020.02.043

García, J. R., Quirós, M., Han, W. M., O'Leary, M. N., Cox, G. N., Nusrat, A., et al. (2019). IFN-γ-tethered Hydrogels Enhance Mesenchymal Stem Cell-Based Immunomodulation and Promote Tissue Repair. *Biomaterials* 220, 119403.

Gauglitz, G. G., and Jeschke, M. G. (2011). Combined Gene and Stem Cell Therapy for Cutaneous Wound Healing. *Mol. Pharmaceutics* 8, 1471–1479. doi:10.1021/mp2001457

Giri, B., Dey, S., Das, T., Sarkar, M., Banerjee, J., and Dash, S. K. (2018). Chronic Hyperglycemia Mediated Physiological Alteration and Metabolic Distortion Leads to Organ Dysfunction, Infection, Cancer Progression and Other Pathophysiological Consequences: An Update on Glucose Toxicity. *Biomed. Pharmacother.* 107, 306–328. doi:10.1016/j.biopha.2018.07.157

Grennan, D. (2019). Diabetic Foot Ulcers. *JAMA* 321, 114. doi:10.1001/jama.2018.18323

Gu, X.-y., Shen, S.-e., Huang, C.-f., Liu, Y.-n., Chen, Y.-c., Luo, L., et al. (2013). Effect of Activated Autologous Monocytes/macrophages on Wound Healing in a Rodent Model of Experimental Diabetes. *Diabetes Res. Clin. Pract.* 102, 53–59. doi:10.1016/j.diabres.2013.08.004

Guo, R., Wan, F., Morimatsu, M., Xu, Q., Feng, T., Yang, H., et al. (2021). Cell Sheet Formation Enhances the Therapeutic Effects of Human Umbilical Cord Mesenchymal Stem Cells on Myocardial Infarction as a Bioactive Material. *Bioactive Mater.* 6, 2999–3012. doi:10.1016/j.bioactmat.2021.01.036

Haertel, E., Joshi, N., Hiebert, P., Kopf, M., and Werner, S. (2018). Regulatory T Cells Are Required for normal and Activin-promoted Wound Repair in Mice. *Eur. J. Immunol.* 48, 1001–1013. doi:10.1002/eji.201747395

Han, C., Zhang, H., Wu, Y., He, X., and Chen, X. (2020). Dual-crosslinked Hyaluronan Hydrogels with Rapid Gelation and High Injectability for Stem Cell protection. *Sci. Rep.* 10, 14997. doi:10.1038/s41598-020-71462-4

Hedhli, J., Konopka, C. J., Schuh, S., Bouvin, H., Cole, J. A., Huntsman, H. D., et al. (2017). Multimodal Assessment of Mesenchymal Stem Cell Therapy for Diabetic Vascular Complications. *Theranostics* 7, 3876–3888. doi:10.7150/thno.19547

Heublein, H., Bader, A., and Giri, S. (2015). Preclinical and Clinical Evidence for Stem Cell Therapies as Treatment for Diabetic Wounds. *Drug Discov. Today* 20, 703–717. doi:10.1016/j.drudis.2015.01.005

Hikabe, O., Hamazaki, N., Nagamatsu, G., Obata, Y., Hirao, Y., Hamada, N., et al. (2016). Reconstitution *In Vitro* of the Entire Cycle of the Mouse Female Germ Line. *Nature* 539, 299–303. doi:10.1038/nature20104

Hochedlinger, K., and Jaenisch, R. (2003). Nuclear Transplantation, Embryonic Stem Cells, and the Potential for Cell Therapy. *N. Engl. J. Med.* 349, 275–286. doi:10.1056/nejmra035397

Hou, C., Shen, L., Huang, Q., Mi, J., Wu, Y., Yang, M., et al. (2013). The Effect of Heme Oxygenase-1 Complexed with Collagen on MSC Performance in the Treatment of Diabetic Ischemic Ulcer. *Biomaterials* 34, 112–120. doi:10.1016/j.biomaterials.2012.09.022

Houlind, K. (2020). Surgical Revascularization and Reconstruction Procedures in Diabetic Foot Ulceration. *Diabetes Metab. Res. Rev.* 36 (Suppl. 1), e3256. doi:10.1002/dmrr.3256

Huang, C., Luo, W., Wang, Q., Ye, Y., Fan, J., Lin, L., et al. (2021). Human Mesenchymal Stem Cells Promote Ischemic Repairment and Angiogenesis of Diabetic Foot through Exosome miRNA-21-5p. *Stem Cel Res.* 52, 102235. doi:10.1016/j.scr.2021.102235

International Diabetes Federation (2019). *Idf Diabetes Atlas*. 9th ed. Brussels, Belgium: International Diabetes Federation.

Ishida, Y., Kuninaka, Y., Nosaka, M., Furuta, M., Kimura, A., Taruya, A., et al. (2019). CCL2-Mediated Reversal of Impaired Skin Wound Healing in Diabetic Mice by Normalization of Neovascularization and Collagen Accumulation. *J. Invest. Dermatol.* 139, 2517–2527. doi:10.1016/j.jid.2019.05.022

Jayasuriya, R., Dhamodharan, U., Karan, A. N., Anandharaj, A., Rajesh, K., and Ramkumar, K. M. (2020). Role of Nrf2 in MALAT1/HIF-1α Loop on the Regulation of Angiogenesis in Diabetic Foot Ulcer. *Free Radic. Biol. Med.* 156, 168–175. doi:10.1016/j.freeradbiomed.2020.05.018

Jiang, X.-Y., Lu, D.-B., and Chen, B. (2012). Progress in Stem Cell Therapy for the Diabetic Foot. *Diabetes Res. Clin. Pract.* 97, 43–50. doi:10.1016/j.diabres.2011.12.011

Jung, J.-A., Yoon, Y.-D., Lee, H.-W., Kang, S.-R., and Han, S.-K. (2018). Comparison of Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells with Healthy Fibroblasts on Wound-Healing Activity of Diabetic Fibroblasts. *Int. Wound J.* 15, 133–139. doi:10.1111/iwj.12849

Kajanne, R., Miettinen, P., Mehlem, A., Leivonen, S.-K., Birrer, M., Foschi, M., et al. (2007). EGF-R Regulates MMP Function in Fibroblasts through MAPK and AP-1 Pathways. *J. Cel. Physiol.* 212, 489–497. doi:10.1002/jcp.21041

Karar, J., and Maity, A. (2011). PI3K/AKT/mTOR Pathway in Angiogenesis. *Front. Mol. Neurosci.* 4, 51. doi:10.3389/fnmol.2011.00051

Kato, J., Kamiya, H., Himeno, T., Shibata, T., Kondo, M., Okawa, T., et al. (2014). Mesenchymal Stem Cells Ameliorate Impaired Wound Healing through Enhancing Keratinocyte Functions in Diabetic Foot Ulcerations on the Plantar Skin of Rats. *J. Diabetes its Complications* 28, 588–595. doi:10.1016/j.jdiacomp.2014.05.003

Kato, Y., Iwata, T., Morikawa, S., Yamato, M., Okano, T., and Uchigata, Y. (2015). Allogeneic Transplantation of an Adipose-Derived Stem Cell Sheet Combined with Artificial Skin Accelerates Wound Healing in a Rat Wound Model of Type 2 Diabetes and Obesity. *Diabetes* 64, 2723–2734. doi:10.2337/db14-1133

Khalid, R. S., Khan, I., Zaidi, M. B., Naeem, N., Haneef, K., Qazi, R. e. M., et al. (2019). IL-7 Overexpression Enhances Therapeutic Potential of Rat Bone Marrow Mesenchymal Stem Cells for Diabetic Wounds. *Wound Rep. Reg.* 27, 235–248. doi:10.1111/wrr.12706

Krishnan, N., Velramar, B., Ramatchandirin, B., Abraham, G. C., Duraisamy, N., Pandiyan, R., et al. (2018). Effect of Biogenic Silver Nanocubes on Matrix Metalloproteinases 2 and 9 Expressions in Hyperglycemic Skin Injury and its Impact in Early Wound Healing in Streptozotocin-Induced Diabetic Mice. *Mater. Sci. Eng. C* 91, 146–152. doi:10.1016/j.msec.2018.05.020

Kuo, Y.-R., Wang, C.-T., Cheng, J.-T., Wang, F.-S., Chiang, Y.-C., and Wang, C.-J. (2011). Bone Marrow-Derived Mesenchymal Stem Cells Enhanced Diabetic Wound Healing through Recruitment of Tissue Regeneration in a Rat Model of Streptozotocin-Induced Diabetes. *Plast. Reconstr. Surg.* 128, 872–880. doi:10.1097/prs.0b013e3182174329

Lanza, R., and Atala, A. (2014). *Essentials of Stem Cell Biology: Third Edition. elsevierdirect.Com*. Amsterdam, Netherlands: Elsevier.

Lee, J. H., Yoon, Y. M., and Lee, S. H. (2017). Hypoxic Preconditioning Promotes the Bioactivities of Mesenchymal Stem Cells via the HIF-1α-GRP78-Akt Axis. *Int. J. Mol. Sci.* 18, 1320. doi:10.3390/ijms18061320

Lee, K.-B., Choi, J., Cho, S.-B., Chung, J.-Y., Moon, E.-S., Kim, N.-S., et al. (2011). Topical Embryonic Stem Cells Enhance Wound Healing in Diabetic Rats. *J. Orthop. Res.* 29, 1554–1562. doi:10.1002/jor.21385

Lepäntalo, M., Apelqvist, J., Setacci, C., Ricco, J. B., de Donato, G., Becker, F., et al. (2011). Chapter V: Diabetic Foot. *Eur. J. Vasc. Endovasc. Surg.* 42 (Suppl. 2), S60–S74. doi:10.1016/S1078-5884(11)60012-9

Li, F., Jia, H., and Yu, C. (2007). ACL Reconstruction in a Rabbit Model Using Irradiated Achilles Allograft Seeded with Mesenchymal Stem Cells or PDGF-B Gene-Transfected Mesenchymal Stem Cells. *Knee Surg. Sports Traumatol. Arthr* 15, 1219–1227. doi:10.1007/s00167-007-0385-x

Li, X., Xie, X., Lian, W., Shi, R., Han, S., Zhang, H., et al. (2018). Exosomes from Adipose-Derived Stem Cells Overexpressing Nrf2 Accelerate Cutaneous Wound Healing by Promoting Vascularization in a Diabetic Foot Ulcer Rat Model. *Exp. Mol. Med.* 50, 29. doi:10.1038/s12276-018-0058-5

Li, X.-Y., Zheng, Z.-H., Li, X.-Y., Guo, J., Zhang, Y., Li, H., et al. (2013). Treatment of Foot Disease in Patients with Type 2 Diabetes Mellitus Using Human Umbilical Cord Blood Mesenchymal Stem Cells: Response and Correction of Immunological Anomalies. *Curr. Pharm. Des.* 19, 4893–4899. doi:10.2174/13816128113199990326

Li, X., Song, Y., Liu, F., Liu, D., Miao, H., Ren, J., et al. (2017). Long Non-coding RNA MALAT1 Promotes Proliferation, Angiogenesis, and Immunosuppressive Properties of Mesenchymal Stem Cells by Inducing VEGF and Ido. *J. Cel. Biochem.* 118, 2780–2791. doi:10.1002/jcb.25927

Liu, G., Ma, H., Qiu, L., Li, L., Cao, Y., Ma, J., et al. (2011). Phenotypic and Functional Switch of Macrophages Induced by Regulatory CD4 + CD25 + T Cells in Mice. *Immunol. Cel Biol.* 89, 130–142. doi:10.1038/icb.2010.70

Liu, J., Zhu, P., Song, P., Xiong, W., Chen, H., Peng, W., et al. (2015). Pretreatment of Adipose Derived Stem Cells with Curcumin Facilitates Myocardial Recovery via Antiapoptosis and Angiogenesis. *Stem Cell Int* 2015, 638153. doi:10.1155/2015/638153

Liu, X.-Q., Duan, L.-S., Chen, Y.-Q., Jin, X.-J., Zhu, N.-N., Zhou, X., et al. (2019). lncRNA MALAT1 Accelerates Wound Healing of Diabetic Mice Transfused with Modified Autologous Blood via the HIF-1α Signaling Pathway. *Mol. Ther. - Nucleic Acids* 17, 504–515. doi:10.1016/j.omtn.2019.05.020

Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., and Dulak, J. (2016). Role of Nrf2/HO-1 System in Development, Oxidative Stress Response and Diseases: an Evolutionarily Conserved Mechanism. *Cell. Mol. Life Sci.* 73, 3221–3247. doi:10.1007/s00018-016-2223-0

Loretelli, C., Ben Nasr, M., Giatsidis, G., Bassi, R., Lancerotto, L., D'Addio, F., et al. (2020). Embryonic Stem Cell Extracts Improve Wound Healing in Diabetic Mice. *Acta Diabetol.* 57, 883–890. doi:10.1007/s00592-020-01500-0

Lv, Y., Ge, L., and Zhao, Y. (2017). Effect and Mechanism of SHED on Ulcer Wound Healing in Sprague-Dawley Rat Models with Diabetic Ulcer. *Am. J. Transl. Res.* 9, 489–498.

Maharlooei, M. K., Bagheri, M., Solhjou, Z., Jahromi, B. M., Akrami, M., Rohani, L., et al. (2011). Adipose Tissue Derived Mesenchymal Stem Cell (AD-MSC) Promotes Skin Wound Healing in Diabetic Rats. *Diabetes Res. Clin. Pract.* 93, 228–234. doi:10.1016/j.diabres.2011.04.018

Mao, A. S., Özkale, B., Shah, N. J., Vining, K. H., Descombes, T., Zhang, L., et al. (2019). Programmable Microencapsulation for Enhanced Mesenchymal Stem Cell Persistence and Immunomodulation. *Proc. Natl. Acad. Sci. USA* 116, 15392–15397. doi:10.1073/pnas.1819415116

Maruyama, K., Asai, J., Ii, M., Thorne, T., Losordo, D. W., and D'Amore, P. A. (2007). Decreased Macrophage Number and Activation lead to Reduced Lymphatic Vessel Formation and Contribute to Impaired Diabetic Wound Healing. *Am. J. Pathol.* 170, 1178–1191. doi:10.2353/ajpath.2007.060018

Mathew, E., Brannon, A. L., Del Vecchio, A., Garcia, P. E., Penny, M. K., Kane, K. T., et al. (2016). Mesenchymal Stem Cells Promote Pancreatic Tumor Growth by Inducing Alternative Polarization of Macrophages. *Neoplasia* 18, 142–151. doi:10.1016/j.neo.2016.01.005

Mathot, F., Shin, A. Y., and Van Wijnen, A. J. (2019). Targeted Stimulation of MSCs in Peripheral Nerve Repair. *Gene* 710, 17–23. doi:10.1016/j.gene.2019.02.078

Matsumura, A., Kubota, T., Taiyoh, H., Fujiwara, H., Okamoto, K., Ichikawa, D., et al. (2013). HGF Regulates VEGF Expression via the C-Met Receptor Downstream Pathways, PI3K/Akt, MAPK and STAT3, in CT26 Murine Cells. *Int. J. Oncol.* 42, 535–542. doi:10.3892/ijo.2012.1728

Matthay, M. A., Goolaerts, A., Howard, J. P., and Woo Lee, J. (2010). Mesenchymal Stem Cells for Acute Lung Injury: Preclinical Evidence. *Crit. Care Med.* 38, S569–S573. doi:10.1097/ccm.0b013e3181f1ff1d

Meng, S.-S., Xu, X.-P., Chang, W., Lu, Z.-H., Huang, L.-L., Xu, J.-Y., et al. (2018). LincRNA-p21 Promotes Mesenchymal Stem Cell Migration Capacity and Survival through Hypoxic Preconditioning. *Stem Cel Res. Ther.* 9, 280. doi:10.1186/s13287-018-1031-x

Mokarram, N., Merchant, A., Mukhatyar, V., Patel, G., and Bellamkonda, R. V. (2012). Effect of Modulating Macrophage Phenotype on Peripheral Nerve Repair. *Biomaterials* 33, 8793–8801. doi:10.1016/j.biomaterials.2012.08.050

Nelson, K. M., Dahlin, J. L., Bisson, J., Graham, J., Pauli, G. F., and Walters, M. A. (2017). The Essential Medicinal Chemistry of Curcumin. *J. Med. Chem.* 60, 1620–1637. doi:10.1021/acs.jmedchem.6b00975

Nicolucci, A., Greenfield, S., and Mattke, S. (2006). Selecting Indicators for the Quality of Diabetes Care at the Health Systems Level in OECD Countries. *Int. J. Qual. Health Care* 18 (Suppl. 1), 26–30. doi:10.1093/intqhc/mzl023

O'Loughlin, A., Kulkarni, M., Creane, M., Vaughan, E. E., Mooney, E., Shaw, G., et al. (2013). Topical Administration of Allogeneic Mesenchymal Stromal Cells Seeded in a Collagen Scaffold Augments Wound Healing and Increases Angiogenesis in the Diabetic Rabbit Ulcer. *Diabetes* 62, 2588–2594. doi:10.2337/db12-1822

Pacific, H., James, P., Smriti, M. K., and Jonathan, G. (2020). Systematic Review and Meta-Analysis of Mouse Models of Diabetes-Associated Ulcers. *BMJ Open Diabetes Res. Care* 8, e000982.

Peck, S. H., Bendigo, J. R., Tobias, J. W., Dodge, G. R., Malhotra, N. R., Mauck, R. L., et al. (2019). Hypoxic Preconditioning Enhances Bone Marrow-Derived Mesenchymal Stem Cell Survival in a Low Oxygen and Nutrient-Limited 3D Microenvironment. *Cartilage* 12, 512. doi:10.1177/1947603519841675

Qin, H., Zhu, X., Zhang, B., Zhou, L., and Wang, W. (2016). Clinical Evaluation of Human Umbilical Cord Mesenchymal Stem Cell Transplantation after Angioplasty for Diabetic Foot. *Exp. Clin. Endocrinol. Diabetes* 124, 497–503. doi:10.1055/s-0042-103684

Regranex (2008). Regranex (Becaplermin) Labeling. Available at: https://www.fda.gov/media/76010.

Robson, M. C. (2003). Cytokine Manipulation of the Wound. *Clin. Plast. Surg.* 30, 57–65. doi:10.1016/s0094-1298(02)00073-1

Rombouts, W. J. C., and Ploemacher, R. E. (2003). Primary Murine MSC Show Highly Efficient Homing to the Bone Marrow but Lose Homing Ability Following Culture. *Leukemia* 17, 160–170. doi:10.1038/sj.leu.2402763

Salo, T., Mäkelä, M., Kylmäniemi, M., Autio-Harmainen, H., and Larjava, H. (1994). Expression of Matrix Metalloproteinase-2 and -9 during Early Human Wound Healing. *Lab. Invest.* 70, 176–182.

Schratzberger, P., Walter, D. H., Rittig, K., Bahlmann, F. H., Pola, R., Curry, C., et al. (2001). Reversal of Experimental Diabetic Neuropathy by VEGF Gene Transfer. *J. Clin. Invest.* 107, 1083–1092. doi:10.1172/jci12188

Shen, L., Zeng, W., Wu, Y.-X., Hou, C.-L., Chen, W., Yang, M.-C., et al. (2013). Neurotrophin-3 Accelerates Wound Healing in Diabetic Mice by Promoting a Paracrine Response in Mesenchymal Stem Cells. *Cel Transpl.* 22, 1011–1021. doi:10.3727/096368912x657495

Shi, H., Xu, X., Zhang, B., Xu, J., Pan, Z., Gong, A., et al. (2017). 3,3′-Diindolylmethane Stimulates Exosomal Wnt11 Autocrine Signaling in Human Umbilical Cord Mesenchymal Stem Cells to Enhance Wound Healing. *Theranostics* 7, 1674–1688. doi:10.7150/thno.18082

Shi, R., Jin, Y., Cao, C., Han, S., Shao, X., Meng, L., et al. (2016). Localization of Human Adipose-Derived Stem Cells and Their Effect in Repair of Diabetic Foot Ulcers in Rats. *Stem Cel Res. Ther.* 7, 155. doi:10.1186/s13287-016-0412-2

Shi, R., Lian, W., Jin, Y., Cao, C., Han, S., Yang, X., et al. (2020). Role and Effect of Vein-Transplanted Human Umbilical Cord Mesenchymal Stem Cells in the Repair of Diabetic Foot Ulcers in Rats. *Acta Biochim. Biophys. Sin (Shanghai)* 52, 620–630. doi:10.1093/abbs/gmaa039

Shomali, N., Gharibi, T., Vahedi, G., Mohammed, R. N., Mohammadi, H., Salimifard, S., et al. (2020). Mesenchymal Stem Cells as Carrier of the Therapeutic Agent in the Gene Therapy of Blood Disorders. *J. Cel. Physiol.* 235, 4120–4134. doi:10.1002/jcp.29324

Silvestre, J.-S., Théry, C., Hamard, G., Boddaert, J., Aguilar, B., Delcayre, A., et al. (2005). Lactadherin Promotes VEGF-dependent Neovascularization. *Nat. Med.* 11, 499–506. doi:10.1038/nm1233

Sireesh, D., Dhamodharan, U., Ezhilarasi, K., Vijay, V., and Ramkumar, K. M. (2018). Association of NF-E2 Related Factor 2 (Nrf2) and Inflammatory Cytokines in Recent Onset Type 2 Diabetes Mellitus. *Sci. Rep.* 8, 5126. doi:10.1038/s41598-018-22913-6

Steed, D. L., Attinger, C., Colaizzi, T., Crossland, M., Franz, M., Harkless, L., et al. (2006). Guidelines for the Treatment of Diabetic Ulcers. *Wound Repair Regen.* 14, 680–692. doi:10.1111/j.1524-475x.2006.00176.x

Stirban, A. (2014). Microvascular Dysfunction in the Context of Diabetic Neuropathy. *Curr. Diab. Rep.* 14, 541. doi:10.1007/s11892-014-0541-x

Storkebaum, E., and Carmeliet, P. (2011). Paracrine Control of Vascular Innervation in Health and Disease. *Acta Physiol(Oxf).* 203, 61–86. doi:10.1111/j.1748-1716.2011.02333.x

Tepper, O. M., Galiano, R. D., Capla, J. M., Kalka, C., Gagne, P. J., Jacobowitz, G. R., et al. (2002). Human Endothelial Progenitor Cells from Type II Diabetics Exhibit Impaired Proliferation, Adhesion, and Incorporation into Vascular Structures. *Circulation* 106, 2781–2786. doi:10.1161/01.cir.0000039526.42991.93

Tombulturk, F. K., Soydas, T., Sarac, E. Y., Tuncdemir, M., Coskunpinar, E., Polat, E., et al. (2019). Regulation of MMP 2 and MMP 9 Expressions Modulated by AP-1 (C-jun) in Wound Healing: Improving Role of *Lucilia sericata* in Diabetic Rats. *Acta Diabetol.* 56, 177–186. doi:10.1007/s00592-018-1237-5

Uchiyama, A., Motegi, S.-i., Sekiguchi, A., Fujiwara, C., Perera, B., Ogino, S., et al. (2017). Mesenchymal Stem Cells-Derived MFG-E8 Accelerates Diabetic Cutaneous Wound Healing. *J. Dermatol. Sci.* 86, 187–197. doi:10.1016/j.jdermsci.2017.02.285

Uchiyama, A., Yamada, K., Ogino, S., Yokoyama, Y., Takeuchi, Y., Udey, M. C., et al. (2014). MFG-E8 Regulates Angiogenesis in Cutaneous Wound Healing. *Am. J. Pathol.* 184, 1981–1990. doi:10.1016/j.ajpath.2014.03.017

Uzun, E., Güney, A., Gönen, Z. B., Özkul, Y., Kafadar İ, H., Günay, M., et al. (2020). Intralesional Allogeneic Adipose-Derived Stem Cells Application in Chronic Diabetic Foot Ulcer: Phase I/2 Safety Study. *Foot Ankle Surg.* 27, 636. doi:10.1016/j.fas.2020.08.002

van Crevel, R., van de Vijver, S., and Moore, D. A. J. (2017). The Global Diabetes Epidemic: what Does it Mean for Infectious Diseases in Tropical Countries? *Lancet Diabetes Endocrinol.* 5, 457–468. doi:10.1016/s2213-8587(16)30081-x

Vig, K., Chaudhari, A., Tripathi, S., Dixit, S., Sahu, R., Pillai, S., et al. (2017). Advances in Skin Regeneration Using Tissue Engineering. *Int. J. Mol. Sci.* 18. doi:10.3390/ijms18040789

Vileikyte, L., Crews, R. T., and Reeves, N. D. (2017). Psychological and Biomechanical Aspects of Patient Adaptation to Diabetic Neuropathy and Foot Ulceration. *Curr. Diab. Rep.* 17, 109. doi:10.1007/s11892-017-0945-5

Villarreal-Ponce, A., Tiruneh, M. W., Lee, J., Guerrero-Juarez, C. F., Kuhn, J., David, J. A., et al. (2020). Keratinocyte-Macrophage Crosstalk by the Nrf2/Ccl2/EGF Signaling Axis Orchestrates Tissue Repair. *Cel Rep.* 33, 108417. doi:10.1016/j.celrep.2020.108417

Vincent, A. M., Callaghan, B. C., Smith, A. L., and Feldman, E. L. (2011). Diabetic Neuropathy: Cellular Mechanisms as Therapeutic Targets. *Nat. Rev. Neurol.* 7, 573–583. doi:10.1038/nrneurol.2011.137

Wan, J., Xia, L., Liang, W., Liu, Y., and Cai, Q. (2013). Transplantation of Bone Marrow-Derived Mesenchymal Stem Cells Promotes Delayed Wound Healing in Diabetic Rats. *J. Diabetes Res.* 2013, 647107. doi:10.1155/2013/647107

Wang, D., Liu, Y., Yang, X., and Zhou, J. (2018). Hypoxic Preconditioning Enhances Cell Hypoxia Tolerance and Correlated lncRNA and mRNA Analysis. *Life Sci.* 208, 46–54. doi:10.1016/j.lfs.2018.07.014

Wang, H., Chen, L., Liu, Y., Luo, B., Xie, N., Tan, T., et al. (2016). Implantation of Placenta-Derived Mesenchymal Stem Cells Accelerates Murine Dermal Wound Closure through Immunomodulation. *Am. J. Transl. Res.* 8, 4912–4921.

Wang, J., Zeng, X. X., Cai, W., Han, Z. B., Zhu, L. Y., Liu, J. Y., et al. (2019). Safety and Efficacy of Placenta-Derived Mesenchymal Stem Cell Treatment for Diabetic Patients with Critical Limb Ischemia: A Pilot Study. *Exp. Clin. Endocrinol. Diabetes* 129, 542. doi:10.1055/a-0978-4972

Wang, M., Crisostomo, P. R., Herring, C., Meldrum, K. K., and Meldrum, D. R. (2006). Human Progenitor Cells from Bone Marrow or Adipose Tissue Produce VEGF, HGF, and IGF-I in Response to TNF by a P38 MAPK-dependent Mechanism. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 291, R880–R884. doi:10.1152/ajpregu.00280.2006

Wang, Z., Li, H., Zhang, D., Liu, X., Zhao, F., Pang, X., et al. (2015). Effect of Advanced Glycosylation End Products on Apoptosis in Human Adipose Tissue-Derived Stem Cells *In Vitro*. *Cell Biosci* 5, 3. doi:10.1186/2045-3701-5-3

Wetzler, C., Kämpfer, H., Stallmeyer, B., Pfeilschifter, J., and Frank, S. (2000). Large and Sustained Induction of Chemokines during Impaired Wound Healing in the Genetically Diabetic Mouse: Prolonged Persistence of Neutrophils and Macrophages during the Late Phase of Repair. *J. Invest. Dermatol.* 115, 245–253. doi:10.1046/j.1523-1747.2000.00029.x

Wysocki, A. B., Staiano-Coico, L., and Grinnell, F. (1993). Wound Fluid from Chronic Leg Ulcers Contains Elevated Levels of Metalloproteinases MMP-2 and MMP-9. *J. Invest. Dermatol.* 101, 64–68. doi:10.1111/1523-1747.ep12359590

Xia, N., Xu, J.-M., Zhao, N., Zhao, Q.-S., Li, M., and Cheng, Z.-F. (2015). Human Mesenchymal Stem Cells Improve the Neurodegeneration of Femoral Nerve in a Diabetic Foot Ulceration Rats. *Neurosci. Lett.* 597, 84–89. doi:10.1016/j.neulet.2015.04.038

Xu, J., Liu, X., Jiang, Y., Chu, L., Hao, H., Liua, Z., et al. (2008). MAPK/ERK Signalling Mediates VEGF-Induced Bone Marrow Stem Cell Differentiation into Endothelial Cell. *J. Cel. Mol. Med.* 12, 2395–2406. doi:10.1111/j.1582-4934.2008.00266.x

Xu, J., Liu, X., Zhao, F., Zhang, Y., and Wang, Z. (2020). HIF1α Overexpression Enhances Diabetic Wound Closure in High Glucose and Low Oxygen Conditions by Promoting Adipose-Derived Stem Cell Paracrine Function and Survival. *Stem Cel Res. Ther.* 11, 148. doi:10.1186/s13287-020-01654-2

Yang, H.-y., Fierro, F., So, M., Yoon, D. J., Nguyen, A. V., Gallegos, A., et al. (2020). Combination Product of Dermal Matrix, Human Mesenchymal Stem Cells, and Timolol Promotes Diabetic Wound Healing in Mice. *Stem Cell Transl. Med.* 9, 1353–1364. doi:10.1002/sctm.19-0380

Yang, J., Chen, Z., Pan, D., Li, H., and Shen, J. (2020). Umbilical Cord-Derived Mesenchymal Stem Cell-Derived Exosomes Combined Pluronic F127 Hydrogel Promote Chronic Diabetic Wound Healing and Complete Skin Regeneration. *Int. J. Nanomedicine* 15, 5911–5926. doi:10.2147/ijn.s249129

Yue, C., Guo, Z., Luo, Y., Yuan, J., Wan, X., and Mo, Z. (2020). c-Jun Overexpression Accelerates Wound Healing in Diabetic Rats by Human Umbilical Cord-Derived Mesenchymal Stem Cells. *Stem Cell Int* 2020, 7430968. doi:10.1155/2020/7430968

Zhang, Q. Z., Su, W. R., Shi, S. H., Wilder-Smith, P., Xiang, A. P., Wong, A., et al. (2010). Human Gingiva-Derived Mesenchymal Stem Cells Elicit Polarization of M2 Macrophages and Enhance Cutaneous Wound Healing. *Stem Cells* 28, 1856–1868. doi:10.1002/stem.503

Zhang, S., Chen, L., Zhang, G., and Zhang, B. (2020). Umbilical Cord-Matrix Stem Cells Induce the Functional Restoration of Vascular Endothelial Cells and Enhance Skin Wound Healing in Diabetic Mice via the Polarized Macrophages. *Stem Cel Res. Ther.* 11, 39. doi:10.1186/s13287-020-1561-x

Zhang, X.-R., Huang, Y.-Z., Gao, H.-W., Jiang, Y.-L., Hu, J.-G., Pi, J.-K., et al. (2020). Hypoxic Preconditioning of Human Urine-Derived Stem Cell-Laden Small Intestinal Submucosa Enhances Wound Healing Potential. *Stem Cel Res. Ther.* 11, 150. doi:10.1186/s13287-020-01662-2

Zhao, L., Guo, Z., Chen, K., Yang, W., Wan, X., Zeng, P., et al. (2020). Combined Transplantation of Mesenchymal Stem Cells and Endothelial Colony-Forming Cells Accelerates Refractory Diabetic Foot Ulcer Healing. *Stem Cell Int* 2020, 8863649. doi:10.1155/2020/8863649

Zhao, Q. S., Xia, N., Zhao, N., Li, M., Bi, C. L., Zhu, Q., et al. (2013). Localization of Human Mesenchymal Stem Cells from Umbilical Cord Blood and Their Role in Repair of Diabetic Foot Ulcers in Rats. *Int. J. Biol. Sci.* 10, 80–89. doi:10.7150/ijbs.7237

Zhao, Y., Zhang, M., Lu, G.-l., Huang, B.-x., Wang, D.-w., Shao, Y., et al. (2020). Hypoxic Preconditioning Enhances Cellular Viability and Pro-angiogenic Paracrine Activity: The Roles of VEGF-A and SDF-1a in Rat Adipose Stem Cells. *Front. Cel Dev. Biol.* 8, 580131. doi:10.3389/fcell.2020.580131

Zhou, S., Wang, Y., Zhang, K., Cao, N., Yang, R., Huang, J., et al. (2020). The Fabrication and Evaluation of a Potential Biomaterial Produced with Stem Cell Sheet Technology for Future Regenerative Medicine. *Stem Cell Int* 2020, 9567362. doi:10.1155/2020/9567362

Zhu, L., Wang, G., Fischbach, S., and Xiao, X. (2017). Suppression of microRNA-205-5p in Human Mesenchymal Stem Cells Improves Their Therapeutic Potential in Treating Diabetic Foot Disease. *Oncotarget* 8, 52294–52303. doi:10.18632/oncotarget.17012

Zhu, L., Zhong, Q., Yang, T., and Xiao, X. (2019). Improved Therapeutic Effects on Diabetic Foot by Human Mesenchymal Stem Cells Expressing MALAT1 as a Sponge for microRNA-205-5p. *Aging* 11, 12236–12245. doi:10.18632/aging.102562

Zubair, M., and Ahmad, J. (2019). Role of Growth Factors and Cytokines in Diabetic Foot Ulcer Healing: A Detailed Review. *Rev. Endocr. Metab. Disord.* 20, 207–217. doi:10.1007/s11154-019-09492-1

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# Rapamycin Promotes the Expansion of Myeloid Cells by Increasing G-CSF Expression in Mesenchymal Stem Cells

*Minghao Li[1,2†], Yanjie Lan[1†], Juan Gao[3], Shengnan Yuan[1], Shuaibing Hou[1], Tengxiao Guo[1], Fei Zhao[1], Yuxia Wang[1], Weiping Yuan[1] and Xiaomin Wang[1,4*]*

[1]State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases, Haihe Laboratory of Cell Ecosystem, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China, [2]Shanghai Blood Center, Shanghai, China, [3]Tianjin Eye Hospital, Tianjin Key Laboratory of Ophthalmology and Visual Science, Tianjin Eye Institute, Clinical College of Ophthalmology, Tianjin Medical University, Nankai University Affiliated Eye Hospital, Tianjin, China, [4]Department of Neuro-oncology, Cancer Center, Beijing Tiantan Hospital, Capital Medical University, Beijing, China

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**Edited by:**
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Uri Ramirez Jarquin,
Max Planck Florida Institute for Neuroscience (MPFI), United States

***Correspondence:**
Xiaomin Wang
xminwang@hotmail.com

[†]These authors have contributed equally to this work and share first authorship

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Rapamycin, also known as sirolimus, an inhibitor of mammalian target of rapamycin (mTOR), is a regulatory kinase responsible for multiple signal transduction pathways. Although rapamycin has been widely used in treating various hematologic diseases, the effects of rapamycin are still not fully understood. Here we found that both oral and intraperitoneal administration of rapamycin led to the expansion of myeloid lineage, while intraperitoneal administration of rapamycin impaired granulocyte differentiation in mice. Rapamycin induced bone marrow mesenchymal stem cells to produce more G-CSF *in vitro* and *in vivo*, and promoted the myeloid cells expansion. Our results thus demonstrated that intraperitoneal administration of rapamycin might promote the expansion of myeloid lineage while impair myeloid cell differentiation *in vivo*.

**Keywords: rapamycin, mTOR, G-CSF, hematopoiesis, myeloid cells**

## INTRODUCTION

Rapamycin (Sirolimus), initially discovered as an antifungal metabolite, produced by *Streptomyces hygroscopicus* from a soil sample of Easter Island (also known as Rapa Nui) (Vézina et al., 1975). It has been reported that rapamycin binds to the immunophilin FK506 binding protein-12 (FKBP12) and inhibits the activation of the mammalian target of rapamycin (mTOR). PI3K-AKT-mTOR pathway was constitutively activated in 60% of AML patients, and the over-activation of PI3K-AKT-mTOR was associated with poor survival of AML patients (Min et al., 2003; Kornblau et al., 2009; Chen et al., 2010; Nepstad et al., 2018; Nepstad et al., 2019). Targeted deletion of mTORC1 in mouse models enhances HSC self-renewal and repopulating properties, and highlights the importance of the individual mTOR-containing complexes at specific stages of HSC homeostasis and haemopoietic lineage commitment and maturation (Malik et al., 2018). Rapamycin, through its inhibition of mTOR, enhanced the anti-tumor effects of doxorubicin on CML cells (Li et al., 2019). *In vitro*, rapamycin promoted CML

**Abbreviation:** mTOR, mammalian target of rapamycin; 4E-BPI, 4E binding protein; S6, p70-kDa S6 ribosomal protein kinase; Rheb1$^{\Delta/\Delta}$, Lyz-Cre; Rheb1$^{fl/fl}$; MSC, Mesenchymal stem cells; G-CSF, Granulocyte-colony-stimulating factor.

cells apoptosis and inhibited CML cell cycle through blocking mTOR signaling pathway (Li et al., 2019). Moreover, rapamycin has been tested alone or in combination with Janus kinase (JAK), ABL protein inhibitors (Tasian et al., 2017), focal adhesion kinase (FAK) or also with cyclin D3 (CCND3) and CDK4/6 inhibitors (Li et al., 2015; Pikman et al., 2017) in xenografts mouse model and cancer cell lines, exhibiting synergistic effects on anti-tumor. Although rapamycin has been developed as a second-generation immunosuppressive and anti-proliferative agent for tumor treatment, the functional impact of rapamycin treatment as an inhibitor of mTORC1 pathway on normal hematopoiesis was not fully understood.

Currently, rapamycin is in early phase clinical trials for a number of malignancies based on the importance of the AKT-mTOR pathway in cancer biology. It has been reported that rapamycin could increase doxorubicin-induced apoptosis of mononuclear cells from non-responder childhood acute lymphoblastic leukemia patients (Avellino et al., 2005) and augment cell sensibility to glucocorticoid induced apoptosis in a subset of primary ALL patients (Wei et al., 2006). The efficacy of rapamycin is also under evaluation in combination with Donor Stem Cell Transplant in adult Ph + B-ALL patients (see www.clinicaltrials.gov/NCT00792948). Despite rapamycin inhibits leukemia cell growth and enhances the anti-tumor effects of chemotherapeutic agents, the usage of rapamycin is limited by its side effects. The intraperitoneal administration of rapamycin induced gastrointestinal discomfort and mouth ulcers, impaired wound healing and elevated circulating triglycerides (Augustine et al., 2007; de Oliveira et al., 2011). However, many of these effects/side effects have not been observed or fully investigated at lower doses of rapamycin in mice (Zhang et al., 2012; Siegmund et al., 2017) or human (Kraig et al., 2018; Yoon et al., 2018). Before rapamycin can be used in leukemia patients, it is critical to understand the undesirable side effects of rapamycin and define a suitable administration of rapamycin to provide its highest benefit while limiting the side effects.

In this study, we explored the effects/side effects of rapamycin on normal hematopoietic cells via inhibiting mTORC1 signaling pathway. We found that oral and intraperitoneal administration of rapamycin increased myeloid cells proliferation through increasing G-CSF expression in MSCs, while intraperitoneal administration of rapamycin impaired neutrophil differentiation.

# MATERIALS AND METHODS

## Mice and Genotyping

Rheb1 conditional deletion mice were generated using the homologous recombination technique to flank exon three of Rheb1 with two LoxP sequences (**Figure 4A**). The mice were then mated with *Lyz-Cre* transgenic mice expressing *Cre* recombinase under the control of the Lyz promoter to delete *Rheb1* in the myeloid cells at the embryonic stage. As expected, half of the offspring were *Rheb1* wild-type ($Rheb1^{fl/fl}$), and the other half were *Rheb1* knockout mice (*Lyz-Cre*; $Rheb1^{fl/fl}$ or $Rheb1^{\Delta/\Delta}$). Mice were maintained at the specific pathogen-free (SPF) animal facility of the State Key Laboratory of Experimental Hematology (SKLEH). All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC), the Institute of Hematology, and Blood Diseases Hospital (CAMS/PUMC). All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize mouse suffering.

## Drugs

Rapamycin (LC Lab, USA) was dissolved in ethanol at 10 mg/ml as stock solution. Its injection solution was further dissolved in PBS with PEG-400 and Tween-80 as cosolvent. The wild type (WT) mice received rapamycin or vehicle (as the control) at dose of 4 mg/kg/day by i. p. every other day for 1 month. The recipient mice received rapamycin at dose of 2 mg/kg/day by p. o. for 4 months after transplantation.

## Flow Cytometry Analysis

20 μL peripheral blood (PB) was obtained from either the tail veins or retroorbital bleeding of mice and diluted with PBE (PBS with 2% fetal bovine serum and 2 mM EDTA). Red blood cells (RBCs) were lysed by red blood cells lysis buffer before staining. Bone marrow (BM) cells were flushed out from tibias, femurs, and ilia by PBE. Cells were stained with antibodies purchased from either eBioscience or BD Bioscience. The cells were stained with the following antibodies: anti-mouse CD3 PE-cy7, anti-mouse B220 FITC, anti-mouse CD11b APC, anti-mouse Ly-6G PE-cy7 for analyzing different lineages. Anti-mouse CD3 biotin, anti-mouse Ly-6G biotin, anti-mouse CD11b biotin, anti-mouse TER-119 biotin, anti-human/mouse B220 biotin, streptavidin FITC, anti-mouse CD117 (c-Kit) APC, anti-mouse Ly-6A/E (Sca-1) PE-Cyanine7, anti-mouse CD45.2 Percp-cy5.5, and anti-mouse CD45.1 PE for HSPCs or anti-mouse CD45.1 FITC, anti-mouse CD45.2 PE, anti-mouse CD11b APC, and anti-mouse Ly-6G (Gr-1) PE-Cyanine7 for neutrophils. All antibodies were purchased from either eBioscience or Invitrogen (United States). To analyze intracellular proteins, $3\times10^6$ BM cells were labeled with surface antibodies, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X100, then washed 2 times with 1 ml cold PBE. Finally, the cells were resuspended with cold PBS supplemented with 25% FBS, and intracellularly stained with anti-bodies: p-S6 (Ser24/244), p-4EBP1 (Thr37/46). Cells were analyzed by BD Canto II flow cytometer. FlowJo software was used to analyze the results.

## Competitive Bone Marrow Transplantation and Analysis

Donor BM cells were isolated from the tibias, femurs and ilia from 8-week-old C57BL/6(B6) mice ($CD45.1^+$). $5\times10^5$ ($CD45.1^+$) together with $5\times10^5$ WBMCs ($CD45.2^+$) were intravenously injected into the lethally irradiated recipient mice ($CD45.2^+$). Then, the reconstituted PB cells were analyzed every 4 weeks after transplantation.

## Quantitative Real-Time PCR (qRT-PCR)

RNA from BM samples was isolated using the RNeasy Mini Kit (QIAGEN, 74106, Germany). cDNA synthesis was

performed using a cDNA reverse transcription kit (Takara, RR047A, Japan) according to the manufacturer's protocol. Quantitative PCR assays were performed in 96-well Micro Amp Fast Optical Reaction Plates (Applied Biosystems, 4344904, United States) using SYBR Green PCR Master Mix (Roche, 04913914001, Switzerland). The signal was detected using the Step-One Plus Real-Time PCR System (QuantStudio5). GAPDH was used as an endogenous control for gene expression assays.

## Isolation of MSCs From Bone and MSCs Culture

Mesenchymal stem cells (MSCs) from the compact bones of mice were obtained as previously described (Zhu et al., 2010). The bone cavities were washed thoroughly at three times using a syringe until the bones become pale to deplete hematopoietic cells from the tibiae and femurs. Hold the humeri, tibiae and femurs with forceps and excise the compact bones carefully into chips of approximately 1–3 $mm^3$ with scissors. The bone chips were transferred into a 25-$cm^2$ plastic culture flask with forceps, then suspend the chips in 3 ml of α-MEM (Hyclone, SH30265.01, United States) containing 10% FBS (Gibco, 16000-044, United States) in the presence of 1 mg/ml of collagenase II (Gibco, 17101015, United States). The chips were digested for 1–2 h in a shaking incubator at 37°C with a shaking speed of 200 rpm. The collagenase digestion was stopped when the bone chips become loosely attached to each other. The digestion medium and released cells were aspirated and discarded. Enzyme-treated bone chips were placed in a 10 $cm^2$ dish containing 6 ml of α-MEM supplemented with 10% FBS. Each replanting was considered a passage. The surfaces marker of MSCs ($Lin^-$ $CD45^-CD31^-CD51^+Sca\text{-}1^+$) were analyzed by Flow cytometry.

## $Lin^-$ c-$kit^+$ ($LK^+$) Isolation and Cocultured With MSCs

BM cells were isolated from the tibias, femurs and ilia of 8-week-old B6. SJL mice. WT $LK^+$ cells were sorted with a c-Kit (CD117) Microbead Kit (MACS, 130-091-224, German) and a Lineage Cell Depletion Kit (MACS, 130-090-858, German) according to the manufacturer's protocol. $5\times10^4$ MSCs were cultured in 24-well plate in a volume of 800 μL α-MEM with 15% FBS and treat with rapamycin or ethanol. After 24 h of culture, the MSCs were cocultured with $2\times10^5$ $LK^+$ cells. After 24 h of coculture, the MSCs were harvested. The $LK^+$ cells were analyzed for the percentage and absolute number of myeloid cells by flow cytometry. For the G-CSF neutralization experiment, G-CSF antibody (R&D, MAB406-SP, United States) was added to the coculture system at 2 nM. After 24 h of coculture. The $LK^+$ cells were analyzed for the percentage and absolute number of myeloid cells by flow cytometry. All cells were incubated at 37°C in a 5% $CO_2$ incubator.

## ELISA

The ELISA was performed using the Mouse G-CSF ELISA Kit (Anoric, TAE-317m, China) according to the manufacturer's protocols. A total of $3\times10^5$ MSC in 200 μL of PBS were frozen and thawed three times and centrifuged at 5,915 rpm (3,000 g) for 10 min, and the liquid supernatants were collected for G-CSF determination. The cell culture medium was concentrated with an ultragentrification device (Millipore, UFC801096, Germany). The ELISA tests were read on a SynergyH four Hybrid Reader at 450 nm.

## Statistical Analyses

GraphPad Prism 8.0 was used for statistical analyses. Every experiment was compared as two groups. All results were analyzed using the unpaired two-tailed Student t-test. $p < 0.05$ was considered significant for all tests. All data are presented as the mean ± standard deviation (SD); Significant difference are indicated with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

# RESULTS

## Intraperitoneal Administration of Rapamycin Increased Myeloid Cells Proliferation

To evaluate the effects of rapamycin in hematopoietic cells, we injected wild type (WT) mice with rapamycin (4 mg/kg/d) or vehicle by i. p. every other day for 1 month and analyzed hematopoietic cell populations in bone marrow (BM) and peripheral blood (PB) by flow cytometry (FACS) (**Figure 1A**). As mTORC1 effectors, the phosphorylation status of S6 and 4 E-BP1 in BM cells was quantitatively evaluated by flow cytometry (FACS) using intracellular staining with antibodies against p-S6 (S240/244) and p-4E-BP1 (T37/46). The fluorescence intensity of p-S6 was decreased in BM cells of mice treated with rapamycin when compared that of controls, while the fluorescence intensity of p-4E-BP1 was not changed in BM cells of mice treated with rapamycin (**Figure 1B**). BM cells and PB cells were collected from mice at 4 weeks after treatment with rapamycin by i. p. and were stained with antibodies to detect hematopoietic stem cells ($LKS^+$, $Lin^-$ c-$Kit^+$ $sca1^+$), hematopoietic progenitor cells ($LKS^-$, $Lin^-$ c-$Kit^+$ $sca1^-$), B cells ($B220^+$), T cells ($CD3^+$) and Myeloid cells (Mac-$1^+$). The absolute number of BM cells in mice treated with rapamycin was equivalent to that of control mice (**Figure 1C**). The percentage of $LKS^+$ and $LKS^-$ cells in mice treated with rapamycin was not altered when compared with controls (**Figure 1D**). The percentage of myeloid cells was increased in BM of mice injected with rapamycin, while the percentage of B cells and T cells was not changed in two groups (**Figure 1E–G**). In consistent with the increase of myeloid cells in BM, the percentage of myeloid cells was also expanded in PB of mice treated with rapamycin (**Figure 1H**). The proportion of B cells in PB of mice injected with rapamycin was slightly decreased contrast with that of control mice (**Figure 1I**), while

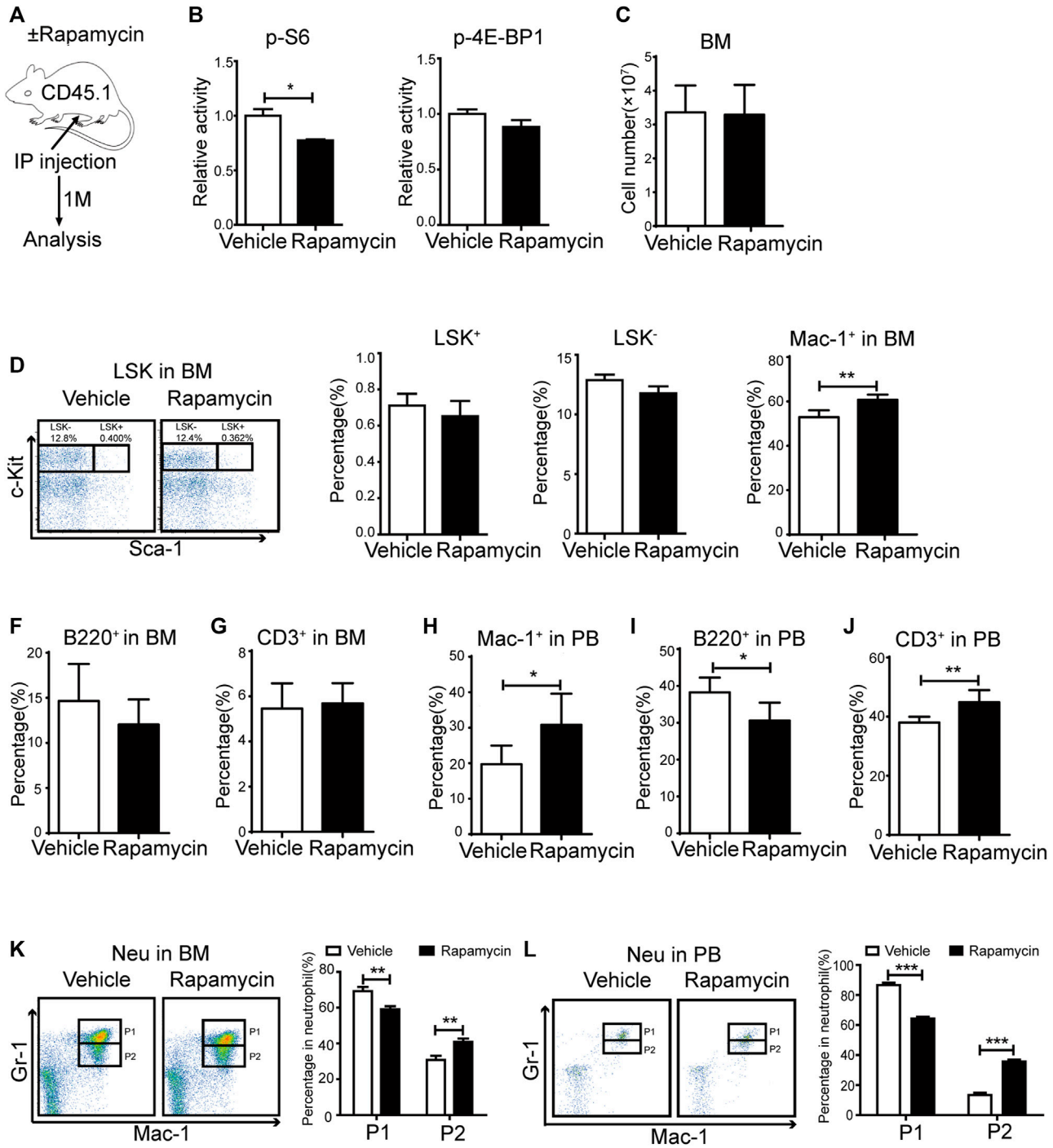


**FIGURE 1 |** Intraperitoneal administration of rapamycin enhanced myeloid cells proliferation **(A)** Experiment design following rapamycin or vehicle treatment, n = 4, days = 30, dose = 4 mg/kg/day **(B)** Fluorescence intensity of p-S6 and p-4E-BP1 in bone marrow **(C)** Absolute number of bone marrow cells **(D)** FACS analysis of LSK$^+$ (Lin$^-$c-Kit$^+$Sca-1$^+$) and LSK$^-$ (Lin$^-$c-Kit$^+$Sca-1$^-$) cells in BM (left panel). Percentage of LSK$^+$ and LSK$^-$ in BM of mice that injected with rapamycin or vehicle (right panel) **(E–G)** Percentage of myeloid cells (CD11b$^+$), B cells (B220$^+$), T cells (CD3$^+$) by FACS in BM in two groups **(H–J)** Percentage of myeloid cells (CD11b$^+$), B cells (B220$^+$), T cells (CD3$^+$) by FACS in PB in two groups **(K)** FACS analysis of neutrophils in BM of mice treated by rapamycin or vehicle, n = 4 (left panel). Percentage of neutrophil subpopulations in two groups; n = 4 (right panel) **(L)** FACS analysis of neutrophils in PB of mice treated by rapamycin or vehicle, n = 4 (left panel). Percentage of neutrophil subpopulations in two groups, n = 4 (right panel).

the percentage of T cells in PB was increased (**Figure 1J**). We further analyzed characteristics of neutrophils by FACS with CD11b and Ly-6G antibodies that have been used as neutrophil subpopulation markers for the identification of myelocytes/promyelocytes, as well as immature and mature neutrophils (Wang et al., 2019). We divided neutrophils into

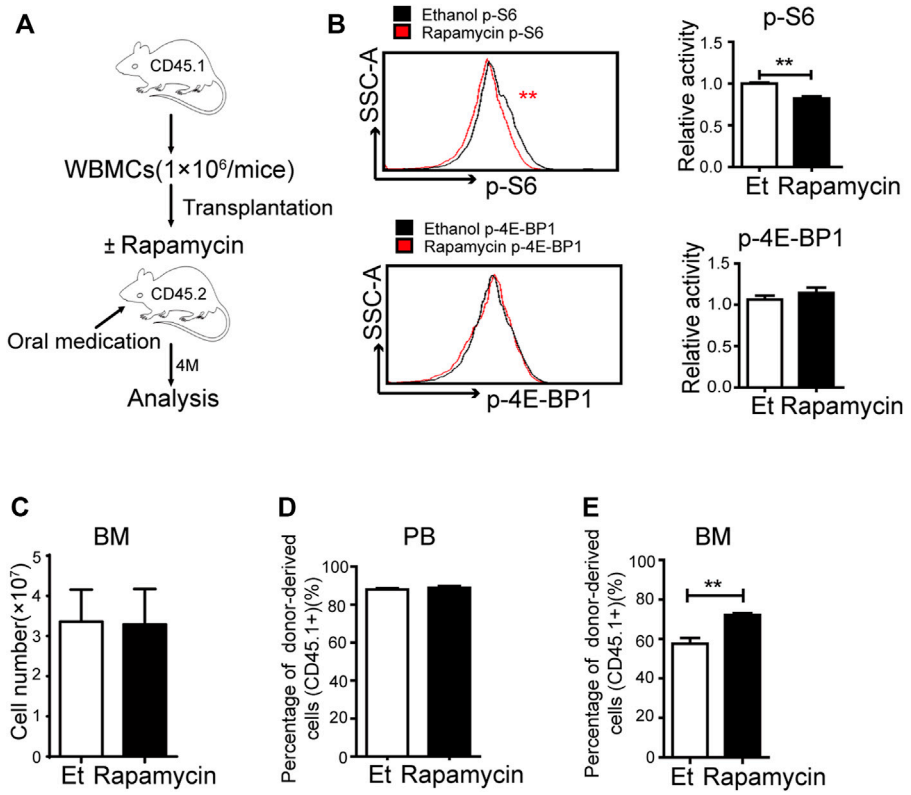


**FIGURE 2 |** Oral administration of rapamycin enhanced BM cells engraftment upon transplantation **(A)** Experiment design with rapamycin treatment of transplantation mouse model with BM cells (CD45.1), n = 3, days = 120, dose = 2 mg/kg/day **(B)** Fluorescence intensity of p-S6 (upper panel) and p-4E-BP1 (lower panel) in BM cells of recipient mice treated by rapamycin or ethanol (control, Et) for 4 months, n = 3 **(C)** Absolute number of bone marrow cells **(D)** Percentage of donor-derived cells ($CD45.1^+$) in the PB in two groups at 4 months; n = 3 **(E)** Percentage of donor-derived cells in the BM in two groups at 4 months; n = 3.

two distinct subpopulations, indicated as the $CD11b^+$ $Ly\text{-}6G^{high}$ population (P1) and the $CD11b^+Ly\text{-}6G^{low}$ population (P2). We found that the percentage of the P2 sub-population was increased, while the percentage of the P1 sub-population was decreased both in the BM and PB of mice treated with rapamycin (**Figure 1K, L**). These data demonstrated that intraperitoneal administration of rapamycin could increase myeloid cells proliferation and impair neutrophil development *in vivo*.

## Oral Administration of Rapamycin Enhanced BM Cells Engraftment Upon Transplantation

To investigate the efficacy of rapamycin in hematopoietic regeneration during transplantation, we transplanted BM cells ($CD45.1^+$) to lethally irradiated mice ($CD45.2^+$) and treated these mice with rapamycin or vehicle by p. o. for 4 months (**Figure 2A**). The recipient mice were sacrificed at 4 months after transplantation and BM cells ($CD45.1^+$) were analyzed. The phosphorylation status of S6 was lower in BM cells of recipient mice treated with rapamycin by p. o. than that of control mice (**Figure 2B**, up panel). The fluorescence status of p-4E-BP1 in BM cells of recipient mice treated with rapamycin by p. o. was equivalent to that of control mice (**Figure 2B**, down panel). We next analyzed the regeneration ability of BM cells and found that the absolute number of BM cells of mice treated with rapamycin by p. o. was equivalent to that of controls (**Figure 2C**). The percentage of donor-derived cells was more than 80% in PB of recipient mice with rapamycin or vehicle by p. o. for 4 months, which indicated the hematopoietic system was recovered by donor BM cells ($CD45.1^+$) in two groups (**Figure 2D**). Notably, the percentage of donor-derived cells in BM of recipient mice treated with rapamycin by p. o. was significantly increased compared with that of controls (**Figure 2E**). It suggested that oral administration of rapamycin could improve regenerated ability of hematopoietic cells upon transplantation.

## Oral Administration of Rapamycin Increased Myeloid Cells Proliferation Upon Transplantation

To clarify whether oral administration of rapamycin has certain effects in hematopoietic cells upon transplantation, we analyzed the proportion of hematopoietic stem/progenitor

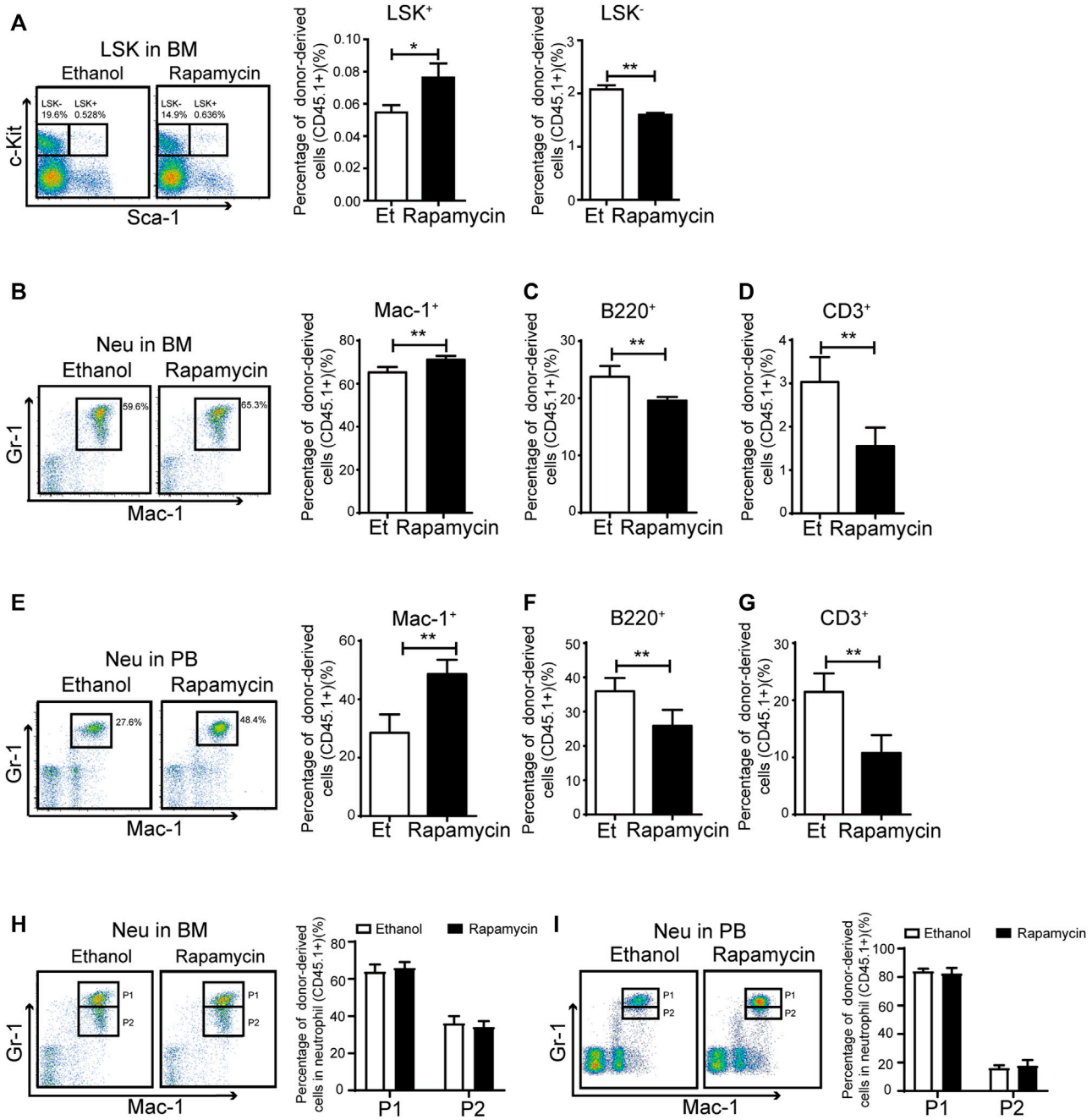


**FIGURE 3 |** Oral administration of rapamycin enhanced myeloid cells proliferation upon transplantation **(A)** Percentage of donor-derived LKS$^+$ (CD45.1) and donor-derived LKS$^-$ (CD45.1) cells in BM of mice (CD45.2) treated by rapamycin or ethanol for 4 months, n = 3 **(B–D)** Percentage of donor-derived myeloid cells (CD45.1), donor-derived B cells (CD45.1) and donor-derived T cells (CD45.1) in BM of two groups, n = 3 **(E–G)** Percentage of donor-derived myeloid cells (CD45.1), donor-derived B cells (CD45.1) and donor-derived T cells (CD45.1) in PB cells of two groups, n = 3 **(H–I)** FACS analysis of donor-derived neutrophils (CD45.1) in BM and PB of mice treated with rapamycin or ethanol for 4 mouths, n = 3.

(HSC/HPCs) populations and several mature populations in BM of recipient mice (CD45.2$^+$) treated with rapamycin or vehicle by p. o. (**Figure 2A**). The percentage of donor-derived LSK$^+$ cells in BM of recipient mice treated with rapamycin by p. o. was equivalent to that of controls (**Figure 3A**, middle panel). However, the percentage of LSK$^-$ cells was decreased in BM of recipient mice treated with rapamycin when compared with that of controls (**Figure 3A**, right panel). The percentage of donor-derived myeloid cells was significantly increased, while the percentage of donor-derived T cells and B cells was

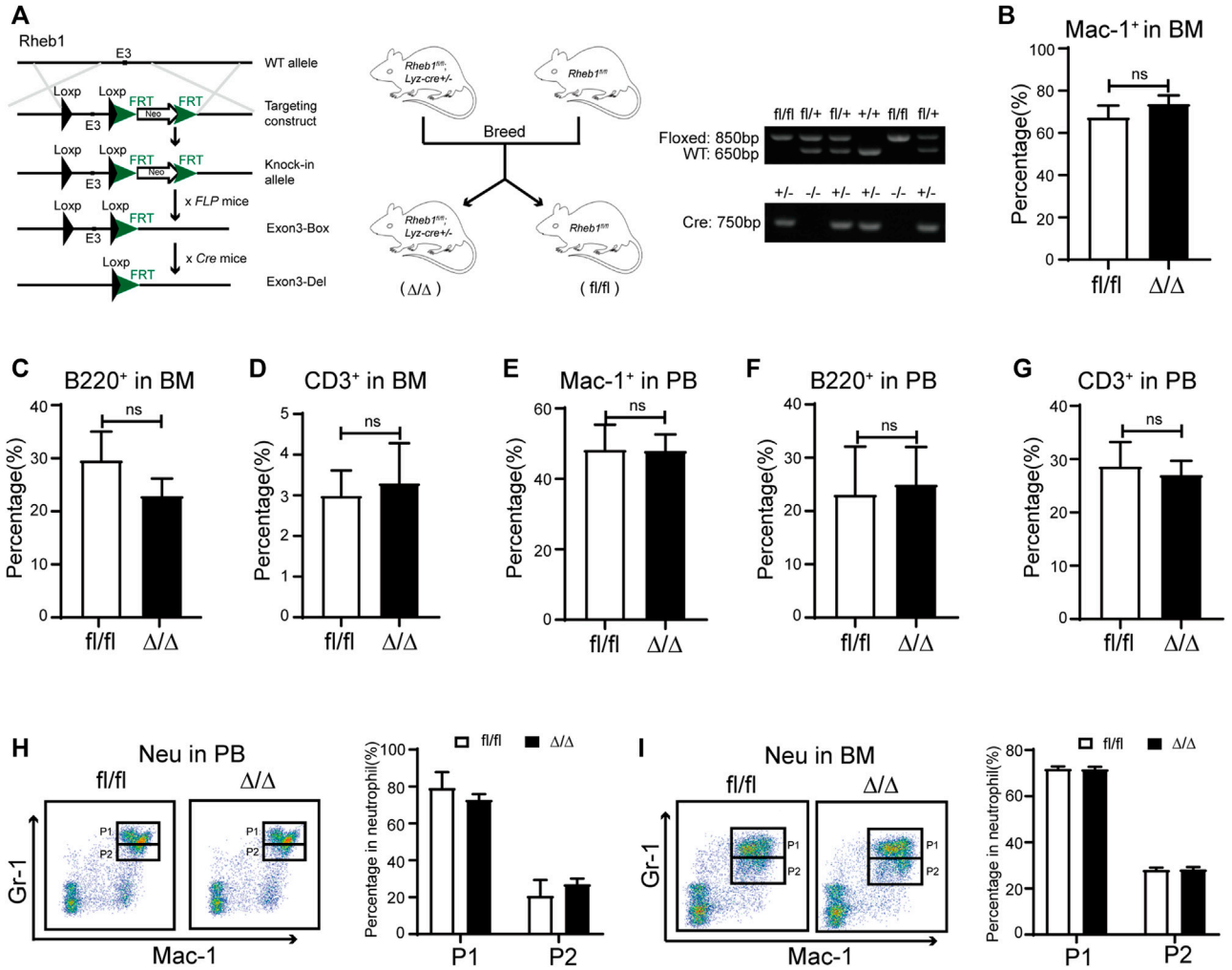


**FIGURE 4 |** Inhibiting mTORC1 signaling in myeloid cells didn't influence its proliferation and differentiation **(A)** Mice model of *Lyz-cre; Rheb1*$^{fl/fl}$ mice (*Rheb1*$^{\Delta/\Delta}$), specific deletion of Rheb1 in the myeloid cells (left and middle panel). The offspring were identified by PCR and analyzed by agarose gel. The size of floxed fragment was 850bp while wild type was 650bp, and the 750 bp segment was amplified from *Cre*$^{+/-}$ (right panel) **(B–D)** Percentage of B cells, T cells and myeloid cells in BM of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$; n = 4 **(E–G)** Percentage of B cells, T cells and myeloid cells in PB of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$; n = 4 **(H)** FACS analysis of neutrophils in PB of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$, n = 4 **(I)** FACS analysis of neutrophils in BM of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$, n = 4.

decreased in BM of recipient mice treated with rapamycin by p. o. when compared with that of controls (**Figure 3B–D**). In consistent with this, the proportion of donor-derived myeloid cells was also increased in PB of recipient mice treated with rapamycin by p. o. for 4 months (**Figure 3E**). The percentage of donor-derived T cells and B cells was decreased in PB of recipient mice treated with rapamycin by p. o. when compared with that of controls (**Figure 3F,G**). Interestingly, the percentage of donor-derived neutrophil P1 sub-population and P2 sub-population was not changed in BM and PB of recipient mice treated with rapamycin by p. o. for 4 months (**Figure 3H,I**). These results indicated that oral administration of rapamycin induced expansion of myeloid cells, but not influenced the development of neutrophils upon transplantation.

## Inhibiting mTORC1 Signaling didn't Influence Myeloid Cells Proliferation and Differentiation

To investigate whether rapamycin impaired neutrophil differentiation through inhibiting mTORC1 signaling pathway, we bred *Lyz-cre; Rheb1*$^{fl/fl}$ mice (*Rheb1*$^{\Delta/\Delta}$), in which Rheb1 was deleted and mTORC1 signaling pathway was inhibited specifically in the myeloid cells. The genotype of *Rheb1*$^{fl/fl}$ and *Lyz-cre; Rheb1*$^{fl/fl}$ mice was verified by genomic PCR (**Figure 4A**). We found the percentage of myeloid cells, T cells and B cells in BM and PB of *Rheb1*$^{\Delta/\Delta}$ mice was equivalent to that of *Rheb1*$^{fl/fl}$ mice (**Figure 4B–G**). The percentage of neutrophil P1 sub-population and P2 sub-population was not changed in BM and PB of *Rheb1*$^{\Delta/\Delta}$ mice when compared with that of *Rheb1*$^{fl/fl}$ mice (**Figure 4H,I**). These data indicated that

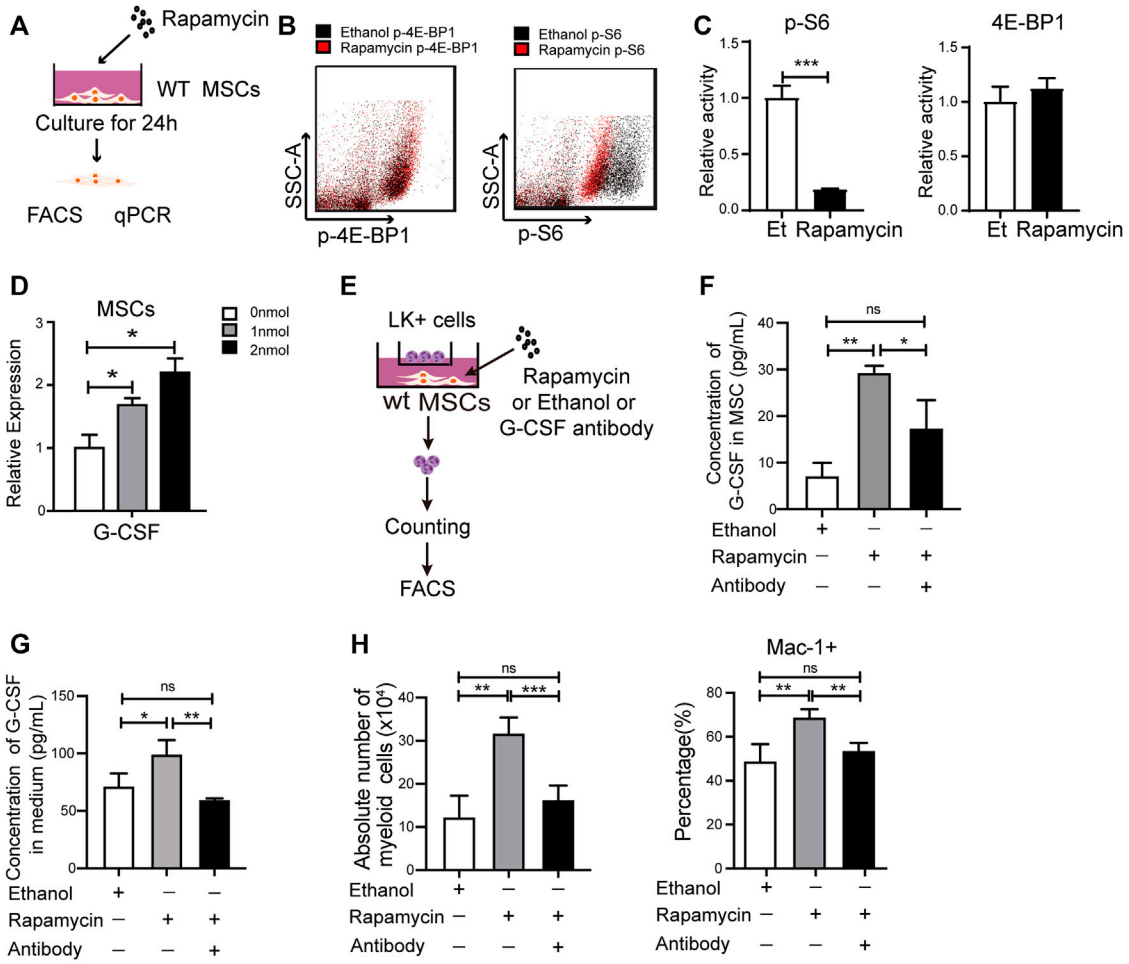


**FIGURE 5 |** Rapamycin increased G-CSF expression in MSCs *in vitro* **(A)** Experiment design for MSCs treated by rapamycin *in vitro* **(B–C)** Fluorescence intensity of p-S6 and p-4E-BP1 in MSCs treated by rapamycin or ethanol *in vitro* **(D)** mRNA expression of G-CSF in MSCs treated by rapamycin or ethanol *in vitro* **(E)** Experiment design for the co-culture of MSC and $LK^+$ cells **(F)** The protein levels of G-CSF in the cell lysates of MSC treated by rapamycin or ethanol **(G)** The G-CSF level in the media of MSC treated by rapamycin or ethanol **(H)** The percentage (Left panel) and absolute number (right panel) of myeloid cells after coculturing with MSCs treated by rapamycin + G-CSF antibody, singel rapamycin, or ethanol.

inhibiting mTORC1 signaling pathway did not influence proliferation and differentiation of myeloid cells.

## Rapamycin Increased G-CSF Expression in MSCs *in vitro*

G-CSF plays an essential role in myeloid expansion and is mainly secreted by Mesenchymal Stem Cells (MSCs) in BM (Lieschke et al., 1994; Boettcher et al., 2014; Lin et al., 2020). To better understand the underlying mechanisms that rapamycin increased expansion of myeloid cells, we isolated MSCs from the BM and cultured MSCs with rapamycin or ethanol treatment for 24 h *in vitro* (**Figure 5A**). We found the phosphorylation of S6 was significantly reduced, while the phosphorylation of 4 E-BP1 was not changed in MSCs treated with rapamycin when compared with that of MSCs treated with ethanol (**Figure 5B,C**). Furthermore, we cultured MSCs with different concentrations of rapamycin and measured the mRNA expression of G-CSF in MSCs by qPCR. We found the mRNA expression of G-CSF was increased sequentially in MSCs after treatment with various doses of rapamycin (**Figure 5D**). We further cocultured $2\times10^5$ $LK^+$ cells with $5\times10^4$ MSCs after treatment with rapamycin or ethanol (**Figure 5E**). We measured the protein expression of G-CSF in the cell lysates of MSC and coculture medium. We found the G-CSF was increased in the MSC treated with rapamycin when compared with that in MSC treated with ethanol (**Figure 5F**). The secreted G-CSF was also higher in medium of MSC treated by rapamycin than that of MSC treated by ethanol (**Figure 5G**). In addition, we found that the percentage and absolute number of myeloid cells was much higher in MSC treated by rapamycin than that in MSC treated by ethanol (**Figure 5H**). Furthermore, we blocked G-CSF by adding G-CSF neutralizing antibody in

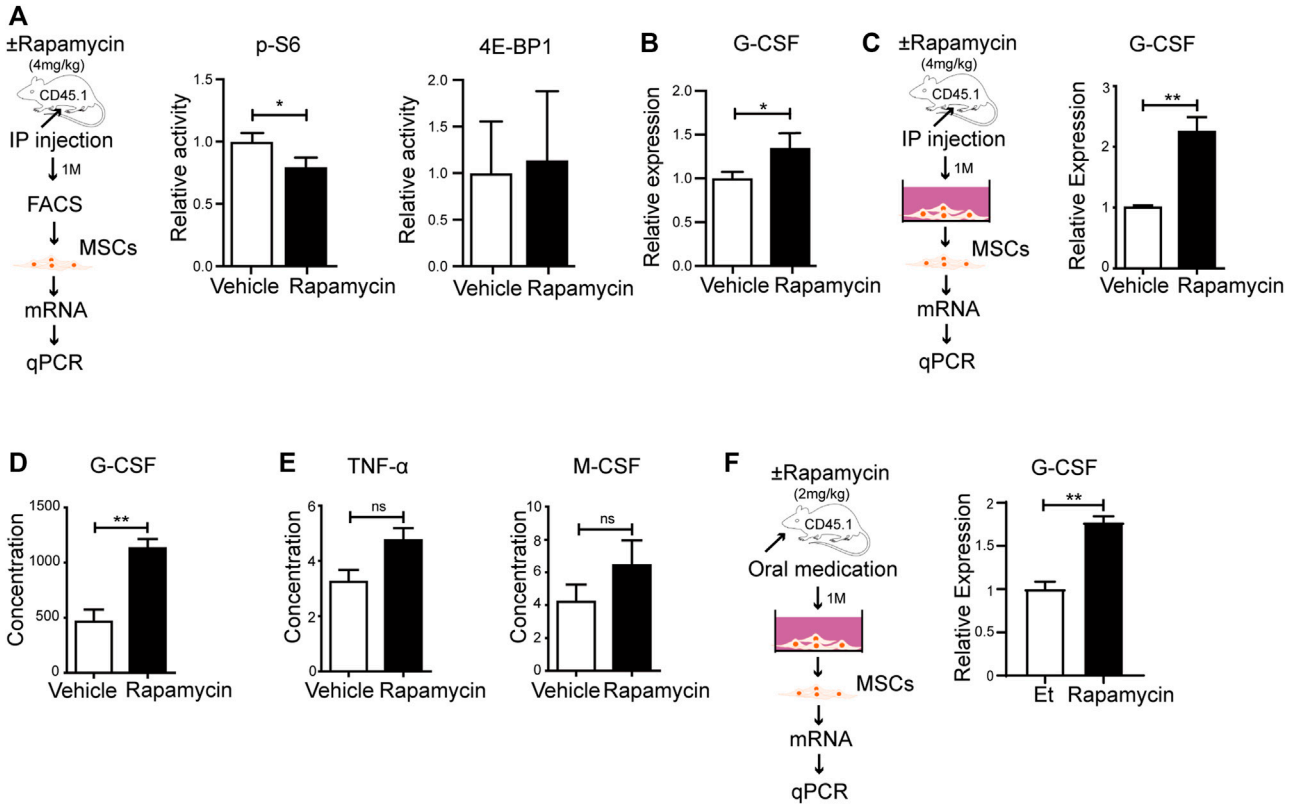


**FIGURE 6 |** Rapamycin prompted MSCs to produce more G-CSF *in vivo* **(A)** Experiment design of MSCs isolated from mice treated with rapamycin or vehicle by i. p., n = 3, days = 30, dose = 4 mg/kg/day (left panel). Fluorescence intensity of p-S6 and p-4E-BP1 in MSCs treated by rapamycin or vehicle by i. p (right and middle panels) **(B)** mRNA relative expression of G-CSF in MSCs from mice treated with rapamycin or vehicle by i. p. **(C)** Experiment design of MSCs cultured *in vitro* (left panel). mRNA relative expression of G-CSF in MSCs after culture (right panel) **(D–E)** Relative expression of G-CSF、TNF-α and M-CSF in serum from mice treated with rapamycin or vehicle by Milliplex multifactor detection technology **(F)** Experiment design of MSCs isolated from mice treated with rapamycin or ethanol by p. o., n = 3, days = 30, dose = 2 mg/kg/day (left panel). Relative mRNA expression of G-CSF in MSCs of two groups (right panel).

the media in which MSCs treated with rapamycin for 24 h and cocultured with $LK^+$ cells. The G-CSF level in the MSC and medium was decreased after adding G-CSF neutralizing antibody when compared with that treated by rapamycin alone (**Figure 5F,G**). We found that blockage of G-CSF significantly decreased the percentage and absolute number of myeloid cells than control (**Figure 5H**). These data suggested that rapamycin might promote myeloid cells expansion by stimulating MSCs to produce G-CSF directly *in vitro*.

## Rapamycin Increased G-CSF Expression in MSCs *in vivo*

To verify if rapamycin could increase the G-CSF expression in MSCs *in vivo*, we injected WT mice with rapamycin or vehicle by i. p. for 1 month. We sorted MSCs from the BM and analyzed phosphorylation level of S6 and 4 E-BP1 by FACS. We found that the phosphorylation of S6 was significantly reduced, while the phosphorylation of 4 E-BP1 was not changed in MSCs from mice treated with rapamycin when compared with that of controls (**Figure 6A**). The mRNA expression of G-CSF was increased in primary MSCs from mice treated with rapamycin when compared with that of controls (**Figure 6B**). Then, we cultured MSCs *in vitro* and analyzed G-CSF expression in MSCs by qPCR. We found that the G-CSF mRNA expression was significantly increased in MSCs from mice treated with rapamycin after culture when compared with that of controls (**Figure 6C**). In order to eliminate the immune response induced by rapamycin, we collected serum from mice treated with rapamycin or vehicle and measured the expression of inflammatory factors in serum. We found that the expression of G-CSF was significantly increased in serum of mice treated with rapamycin when compared with that of control mice (**Figure 6D**). However, TNF-α and M-CSF expression was not changed in serum of mice treated with rapamycin (**Figure 6E**). To assess the G-CSF expression in MSCs from mice treated with rapamycin or ethanol by p. o., MSCs were sorted and cultured *in vitro*. We found the G-CSF mRNA expression was significantly increased in MSCs from mice treated with rapamycin by p. o. when compared with that of controls (**Figure 6F**). These data indicated that rapamycin

promote myeloid cells expansion through increasing G-CSF expression in MSCs.

## DISCUSSION

The defects of bone marrow monocyte/macrophage differentiation caused by mTOR deficiency has been systematically studied with various mouse models. As a mTOR inhibitor, rapamycin could inhibit proliferation and impair differentiation of megakaryocyte and dendritic cell (Guo et al., 2013). In this study, by using murine models, we found that both intraperitoneal and oral administration of rapamycin led to remarkable expansion of myeloid lineage, which was caused by more G-CSF secreted by MSCs. Our data suggested that rapamycin influenced normal hematopoiesis by inhibiting mTOR signaling pathway.

Reducing mTORC1 activity was shown to lead to neutrophils immaturation (Wang et al., 2019). Interestingly, we found that intraperitoneal administration of rapamycin impaired neutrophil development, while oral administration of rapamycin did not influence the cells development. Since the absorption, distribution, and the circulating and/or tissue concentrations of rapamycin are subjected to the given dose and route (Magari et al., 1997), the degree of oral bioavailability and resultant circulating and/or tissue rapamycin concentration might be lower than intraperitoneal administration of rapamycin. It is conceivable that a higher dose of rapamycin treatment could completely inhibit mTOR. But a lower dose of rapamycin could potentially improve the tissue metabolic milieu by normalizing, and not completely inhibiting mTOR signaling (den Hartigh et al., 2018). Furthermore, this lower dose could also limit the complex feedback mechanisms involved in the activation of IRS1-PI3K-AKT. We found that p-S6 was reduced significantly in BM cells of recipient mice after treatment with rapamycin by p. o. or i. p. As a downstream target of mTORC1, S6K could negatively regulate mTORC2 (Malik et al., 2018), while mTORC2 activates AKT signaling pathway and promotes cell proliferation (Jacinto et al., 2006; Yang et al., 2006). The reduction in S6K phosphorylation/activation releases S6K's inhibition of mTORC2, thus increases the phosphorylation level of $AKT^{S473}$. This may cause an aberration in the S6K-mediated negative feedback loop which regulates mTORC2 activity, and further affects cell survival (Guo et al., 2013; Malik et al., 2018). Our findings suggested that oral administration of rapamycin by p. o. might have similar effects on proliferation of myeloid lineage cells when compared with the intraperitoneal administration of rapamycin by i. p., while oral administration of rapamycin by p. o. had little adverse effects on neutrophil differentiation through the feedback loop. Moreover, the oral administration of rapamycin might affect myeloid lineage cells proliferation by both mTORC1 and mTORC2 signaling pathways.

Rheb1 acts as a key upstream activator of mTOR to play vital roles in maintaining proper hematopoiesis and myeloid differentiation (Aspuria and Tamanoi 2004). Our previous studies found that the proliferation of myeloid lineage cells in BM and PB were increase in HSCs of *Vav1-Cre;Rheb1*$^{fl/fl}$ mice (Wang et al., 2019; Gao et al., 2021). In addition, mTOR-deficiency impaired GMPs differentiation into the monocyte/macrophage lineage (Zhao et al., 2018). In our data, the percentage of myeloid lineage cells was equivalent in BM and PB of *Rheb1*$^{fl/fl}$ mice and *Lyz-cre; Rheb1*$^{fl/fl}$ mice, in which Rheb1 was specifically deleted in monocytic lineage stage. Rheb1 deletion in mature monocytes/macrophages doesn't affect the differentiation of myeloid lineage cells. Thus, we suspected that rapamycin might have influences on GMPs/HPCs/HSCs rather than the terminal monocytes/macrophages through mTORC1 signaling pathway.

In our study, we found the phosphorylation of S6 was significantly reduced in MSCs treated with rapamycin. It has been known that rapamycin could decrease the phosphorylation of S6, eIF4B and eEF2 by blocking the kinase activity of mTORC1, while it did not influence the expression of S6, eIF4B and eEF2 (Huo et al., 2011). So we think rapamycin affect MSCs by inhibiting the activity of mTORC1. The mechanisms by which mTOR inhibition protects from stem cell exhaustion and aging have been associated with secretion of major senescence-associated cytokines, such as SCF (Aspuria and Tamanoi 2004). In our studies, rapamycin significantly increased G-CSF expression in MSCs. G-CSF is secreted by bone marrow stromal cells, endothelial cells, macrophages, and fibroblast cells (Boettcher et al., 2014). G-CSF plays an important role in regulating immune cell number and function in allografts. Previous study indicated that G-CSF modulated NK subpopulations in PB and BM (Yu et al., 2018). G-CSF limited the IFN-γ signaling in T cells and induce immune tolerance (Franzke et al., 2003; Chang et al., 2019). In addition, G-CSF decreases the production of TNF-α, IL-2, IFN-γ, and modulates immune responses (Franzke et al., 2003). Moreover, G-CSF is the most important regulator that drives hematopoiesis of stem cells to differentiate into common myeloid progenitors and granulocyte/macrophage progenitors (Basu et al., 2002). Our studies indicate that rapamycin may promote the expansion of myeloid lineage cells in BM and PB through regulating G-CSF expression in MSCs by blocking mTORC1 signaling pathway, while the increased G-CSF in MSCs might be not robust enough to rescue the inhibition of myeloid cells differentiation induced by intraperitoneal administration of rapamycin *in vivo*.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC), the Institute of Hematology, and Blood Diseases Hospital (CAMS/PUMC).

## AUTHOR CONTRIBUTIONS

XW and WY conceived the project, supervised the research and revised the paper. ML and YL performed most of the experiments, analyzed the data, and wrote the manuscript. JG, SY, SH, TG, FZ and YW assisted with the mouse experiments, flow cytometry analysis and data processing. ML and JG contributed to the data analyses and paper discussion. All authors read and approved the final manuscript.

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## REFERENCES

Aspuria, P.-J., and Tamanoi, F. (2004). The Rheb Family of GTP-Binding Proteins. *Cell Signal.* 16 (10), 1105–1112. doi:10.1016/j.cellsig.2004.03.019

Augustine, J. J., Bodziak, K. A., and Hricik, D. E. (2007). Use of Sirolimus in Solid Organ Transplantation. *Drugs* 67 (3), 369–391. doi:10.2165/00003495-200767030-00004

Avellino, R., Romano, S., Parasole, R., Bisogni, R., Lamberti, A., Poggi, V., et al. (2005). Rapamycin Stimulates Apoptosis of Childhood Acute Lymphoblastic Leukemia Cells. *Blood* 106 (4), 1400–1406. doi:10.1182/blood-2005-03-0929

Basu, S., Dunn, A., and Ward, A. (2002). G-CSF: Function and Modes of Action (Review). *Int. J. Mol. Med.* 10 (1), 3–10. doi:10.3892/ijmm.10.1.3

Boettcher, S., Gerosa, R. C., Radpour, R., Bauer, J., Ampenberger, F., Heikenwalder, M., et al. (2014). Endothelial Cells Translate Pathogen Signals into G-CSF-Driven Emergency Granulopoiesis. *Blood* 124 (9), 1393–1403. doi:10.1182/blood-2014-04-570762

Chang, Y.-J., Zhao, X.-Y., and Huang, X.-J. (2019). Granulocyte Colony-Stimulating Factor-Primed Unmanipulated Haploidentical Blood and Marrow Transplantation. *Front. Immunol.* 10, 2516. doi:10.3389/fimmu.2019.02516

Chen, W., Drakos, E., Grammatikakis, I., Schlette, E. J., Li, J., Leventaki, V., et al. (2010). mTOR Signaling Is Activated by FLT3 Kinase and Promotes Survival of FLT3- Mutated Acute Myeloid Leukemia Cells. *Mol. Cancer* 9, 292. doi:10.1186/1476-4598-9-292

de Oliveira, M. A., Martins e Martins, F., Wang, Q., Sonis, S., Demetri, G., George, S., et al. (2011). Clinical Presentation and Management of mTOR Inhibitor-Associated Stomatitis. *Oral Oncol.* 47 (10), 998–1003. doi:10.1016/j.oraloncology.2011.08.009

den Hartigh, L. J., Goodspeed, L., Wang, S. A., Kenerson, H. L., Omer, M., O'Brien, K. D., et al. (2018). Chronic Oral Rapamycin Decreases Adiposity, Hepatic Triglycerides and Insulin Resistance in Male Mice Fed a Diet High in Sucrose and Saturated Fat. *Exp. Physiol.* 103 (11), 1469–1480. doi:10.1113/ep087207

Franzke, A., Piao, W., Lauber, J., Gatzlaff, P., Könecke, C., Hansen, W., et al. (2003). G-CSF as Immune Regulator in T Cells Expressing the G-CSF Receptor: Implications for Transplantation and Autoimmune Diseases. *Blood* 102 (2), 734–739. doi:10.1182/blood-2002-04-1200

Gao, J., Hou, S., Yuan, S., Wang, Y., Gao, Y., Sun, X., et al. (2021). Rheb1-Deficient Neutrophils Promote Hematopoietic Stem/Progenitor Cell Proliferation via Mesenchymal Stem Cells. *Front. Cel Dev. Biol.* 9, 650599. doi:10.3389/fcell.2021.650599

Guo, F., Zhang, S., Grogg, M., Cancelas, J. A., Varney, M. E., Starczynowski, D. T., et al. (2013). Mouse Gene Targeting Reveals an Essential Role of mTOR in Hematopoietic Stem Cell Engraftment and Hematopoiesis. *Haematologica* 98 (9), 1353–1358. doi:10.3324/haematol.2012.080424

Huo, Y., Iadevaia, V., and Proud, C. G. (2011). Differing Effects of Rapamycin and mTOR Kinase Inhibitors on Protein Synthesis. *Biochem. Soc. Trans.* 39 (2), 446–450. doi:10.1042/bst0390446

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., et al. (2006). SIN1/MIP1 Maintains Rictor-mTOR Complex Integrity and Regulates Akt Phosphorylation and Substrate Specificity. *Cell* 127 (1), 125–137. doi:10.1016/j.cell.2006.08.033

Kornblau, S. M., Tibes, R., Qiu, Y. H., Chen, W., Kantarjian, H. M., Andreeff, M., et al. (2009). Functional Proteomic Profiling of AML Predicts Response and Survival. *Blood* 113 (1), 154–164. doi:10.1182/blood-2007-10-119438

Kraig, E., Linehan, L. A., Liang, H., Romo, T. Q., Liu, Q., Wu, Y., et al. (2018). A Randomized Control Trial to Establish the Feasibility and Safety of Rapamycin Treatment in an Older Human Cohort: Immunological, Physical Performance, and Cognitive Effects. *Exp. Gerontol.* 105, 53–69. doi:10.1016/j.exger.2017.12.026

Li, H., Kong, X., Cui, G., Ren, C., Fan, S., Sun, L., et al. (2015). Rapamycin Restores P14, P15 and P57 Expression and Inhibits the mTOR/p70S6K Pathway in Acute Lymphoblastic Leukemia Cells. *Int. J. Hematol.* 102 (5), 558–568. doi:10.1007/s12185-015-1858-1

Li, J., Liu, W., Hao, H., Wang, Q., and Xue, L. (2019). Rapamycin Enhanced the Antitumor Effects of Doxorubicin in Myelogenous Leukemia K562 Cells by Downregulating the mTOR/p70S6K Pathway. *Oncol. Lett.* 18 (3), 2694–2703. doi:10.3892/ol.2019.10589

Lieschke, G., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., et al. (1994). Mice Lacking Granulocyte colony-stimulating Factor Have Chronic Neutropenia, Granulocyte and Macrophage Progenitor Cell Deficiency, and Impaired Neutrophil Mobilization. *Blood* 84 (6), 1737–1746. doi:10.1182/blood.v84.6.1737.bloodjournal8461737

Lin, L., Hwang, B.-J., Li, N., Googe, P., Diaz, L. A., Miao, E., et al. (2020). Non-Cell-Autonomous Activity of the Hemidesmosomal Protein BP180/Collagen XVII in Granulopoiesis in Humanized NC16A Mice. *J.I.* 205 (10), 2786–2794. doi:10.4049/jimmunol.2000784

Magari, S. R., Rivera, V. M., Iuliucci, J. D., Gilman, M., and Cerasoli, F., Jr. (1997). Pharmacologic Control of a Humanized Gene Therapy System Implanted into Nude Mice. *J. Clin. Invest.* 100 (11), 2865–2872. doi:10.1172/jci119835

Malik, N., Sansom, O. J., and Michie, A. M. (2018). The Role of mTOR-Mediated Signals during Haemopoiesis and Lineage Commitment. *Biochem. Soc. Trans.* 46 (5), 1313–1324. doi:10.1042/bst20180141

Min, Y. H., Eom, J. I., Cheong, J. W., Maeng, H. O., Kim, J. Y., Jeung, H. K., et al. (2003). Constitutive Phosphorylation of Akt/PKB Protein in Acute Myeloid Leukemia: its Significance as a Prognostic Variable. *Leukemia* 17 (5), 995–997. doi:10.1038/sj.leu.2402874

Nepstad, I., Hatfield, K. J., Tvedt, T. H. A., Reikvam, H., and Bruserud, Ø. (2018). Clonal Heterogeneity Reflected by PI3K-AKT-mTOR Signaling in Human Acute Myeloid Leukemia Cells and its Association with Adverse Prognosis. *Cancers (Basel)* 10 (9), 1. doi:10.3390/cancers10090332

Nepstad, I., Hatfield, K. J., Grønningsæter, I. S., Aasebø, E., Hernandez-Valladares, M., Hagen, K. M., et al. (2019). Effects of Insulin and Pathway Inhibitors on the PI3K-Akt-mTOR Phosphorylation Profile in Acute Myeloid Leukemia Cells. *Sig Transduct Target. Ther.* 4, 20. doi:10.1038/s41392-019-0050-0

Pikman, Y., Alexe, G., Roti, G., Conway, A. S., Furman, A., Lee, E. S., et al. (2017). Synergistic Drug Combinations with a CDK4/6 Inhibitor in T-Cell Acute Lymphoblastic Leukemia. *Clin. Cancer Res.* 23 (4), 1012–1024. doi:10.1158/1078-0432.ccr-15-2869

Siegmund, S. E., Yang, H., Sharma, R., Javors, M., Skinner, O., Mootha, V., et al. (2017). Low-dose Rapamycin Extends Lifespan in a Mouse Model of mtDNA

Depletion Syndrome. *Hum. Mol. Genet.* 26 (23), 4588–4605. doi:10.1093/hmg/ddx341

Tasian, S. K., Teachey, D. T., Li, Y., Shen, F., Harvey, R. C., Chen, I.-M., et al. (2017). Potent Efficacy of Combined PI3K/mTOR and JAK or ABL Inhibition in Murine Xenograft Models of Ph-like Acute Lymphoblastic Leukemia. *Blood* 129 (2), 177–187. doi:10.1182/blood-2016-05-707653

Vézina, C., Kudelski, A., and Sehgal, S. N. (1975). Rapamycin (AY-22,989), a New Antifungal Antibiotic. I. Taxonomy of the Producing Streptomycete and Isolation of the Active Principle. *J. Antibiot.* 28 (10), 721–726. doi:10.7164/antibiotics.28.721

Wang, X., Gao, Y., Gao, J., Li, M., Zhou, M., Wang, J., et al. (2019). Rheb1 Loss Leads to Increased Hematopoietic Stem Cell Proliferation and Myeloid-Biased Differentiation *In Vivo*. *Haematologica* 104 (2), 245–255. doi:10.3324/haematol.2018.194811

Wei, G., Twomey, D., Lamb, J., Schlis, K., Agarwal, J., Stam, R. W., et al. (2006). Gene Expression-Based Chemical Genomics Identifies Rapamycin as a Modulator of MCL1 and Glucocorticoid Resistance. *Cancer Cell* 10 (4), 331–342. doi:10.1016/j.ccr.2006.09.006

Yang, Q., Inoki, K., Ikenoue, T., and Guan, K.-L. (2006). Identification of Sin1 as an Essential TORC2 Component Required for Complex Formation and Kinase Activity. *Genes Dev.* 20 (20), 2820–2832. doi:10.1101/gad.1461206

Yoon, H.-Y., Hwang, J. J., Kim, D. S., and Song, J. W. (2018). Efficacy and Safety of Low-Dose Sirolimus in Lymphangioleiomyomatosis. *Orphanet J. Rare Dis.* 13 (1), 204. doi:10.1186/s13023-018-0946-8

Yu, X. X., Han, T. T., Xu, L. L., Chang, Y. J., Huang, X. J., and Zhao, X. Y. (2018). Effect of the *In Vivo* Application of Granulocyte colony-stimulating Factor on NK Cells in Bone Marrow and Peripheral Blood. *J. Cell. Mol. Medi* 22 (6), 3025–3034. doi:10.1111/jcmm.13539

Zhang, Z., Wu, X., Duan, J., Hinrichs, D., Wegmann, K., Zhang, G. L., et al. (2012). Low Dose Rapamycin Exacerbates Autoimmune Experimental Uveitis. *PLoS One* 7 (5), e36589. doi:10.1371/journal.pone.0036589

Zhao, Y., Shen, X., Na, N., Chu, Z., Su, H., Chao, S., et al. (2018). mTOR Masters Monocyte Development in Bone Marrow by Decreasing the Inhibition of STAT5 on IRF8. *Blood* 131 (14), 1587–1599. doi:10.1182/blood-2017-04-777128

Zhu, H., Guo, Z.-K., Jiang, X.-X., Li, H., Wang, X.-Y., Yao, H.-Y., et al. (2010). A Protocol for Isolation and Culture of Mesenchymal Stem Cells from Mouse Compact Bone. *Nat. Protoc.* 5 (3), 550–560. doi:10.1038/nprot.2009.238

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# Periosteal Skeletal Stem Cells and Their Response to Bone Injury

*Nian Zhang, Liru Hu, Zhiwei Cao, Xian Liu and Jian Pan**

*State Key Laboratory of Oral Diseases, Department of Oral and Maxillofacial Surgery, National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, China*

Bone exhibits remarkable self-repair ability without fibrous scars. It is believed that the robust regenerative capacity comes from tissue-resident stem cells, such as skeletal stem cells (SSCs). Roughly, SSC has two niches: bone marrow (BM) and periosteum. BM-SSCs have been extensively studied for years. In contrast, our knowledge about periosteal SSCs (P-SSCs) is quite limited. There is abundant clinical evidence for the presence of stem cell populations within the periosteum. Researchers have even successfully cultured "stem-like" cells from the periosteum *in vitro*. However, due to the lack of effective markers, it is difficult to evaluate the stemness of real P-SSCs *in vivo*. Recently, several research teams have developed strategies for the successful identification of P-SSCs. For the first time, we can assess the stemness of P-SSCs from visual evidence. BM-SSCs and P-SSCs not only have much in common but also share distinct properties. Here, we provide an updated review of P-SSCs and their particular responses to bone injury.

**Keywords: skeletal stem cell, periosteum, fate decision, lineage tracing, bone repair**

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*The University of Auckland, New Zealand*

****Correspondence:***
*Jian Pan*
*jianpancn@scu.edu.cn*

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## INTRODUCTION

The periosteum, a specialized membranous structure covering the surface of cortical bones, is a crucial component of diaphysis growth during intramembranous and endochondral bone development (Olsen et al., 2000). Bone tissue is constantly remodeling throughout the lifetime. In addition to the essential function of nourishing bones, the periosteum also maintains bone homeostasis by forming bones directly underneath it (Ferretti and Mattioli-Belmonte, 2014). Although the bones are formed by either intramembranous ossification or endochondral ossification, there appears to be a difference in periosteal structure between the two. Histologically, the periosteum consists of two layers serving as the attachment site of skeletal muscles (Allen et al., 2004). The outer layer (fibrous layer) comprises collagen, elastin, and scarce cells (Buckwalter and Cooper, 1987; Squier et al., 1990; Dwek, 2010). In addition, it also contains a high density of distinct microvascular networks called the "umbilical cord of bone" (Chanavaz, 1995) and linear neuron fibers (Mach et al., 2002; Matsuo et al., 2019). The inner layer (cellular layer) contains a slew of fibroblast-like cells. It is a highly vascularized structure with a high density of microvessels (Dwek, 2010), also known as the cambium layer, which is thicker in early life and then becomes thinner with age (Uddströmer, 1978).

Numerous studies from clinical trials and animal models have shown the osteogenic capacity of the adult periosteum. Periosteal grafts have been successfully used to reconstruct large quantities of bone tissue with high quality in the treatment of pseudarthrosis or infected sites (Masquelet et al., 1988), large bone defects (Lapierre et al., 1991; Gallardo-Calero et al., 2019), fracture non-union (Jaloux et al., 2020), and osteoradionecrosis (Yachouh et al., 2010). The periosteal reaction is another example. The periosteum may be elevated from the cortex in response to various insults, such as

trauma, infection, and tumors. As a result, the periosteum can form new laminated or onion skin-like bones (Rana et al., 2009), which are also critical for mechanical loading. Femurs covered with periosteum showed significantly higher bone strength than the periosteum-stripped ones (Yiannakopoulos et al., 2008). The ablation of periosteal skeletal stem cells (P-SSCs) severely interfered with routine maintenance of bone homeostasis and mechanical loading-induced bone formation (Moore et al., 2018).

In bone repair, the regenerated cells may come from two resources, one from the circulation and the other from local tissues. Whether circulating "stem cells" are involved in the production of osteoblasts has not been proven until recently. Ransom et al. (2018) surgically connected the blood vessels from green fluorescent protein (GFP)-labeled and non-GFP-labeled littermate mice so that they shared a common circulation system. Then, the non-GFP mice were treated with distraction osteogenesis (DO) surgery. Twenty-nine days after surgery, no GFP + cells were detected in the distraction callus (Ransom et al., 2018). In another study, fractured femurs from a genetically labeled donor were transplanted into the renal capsule of a wild-type host. After 14 days, the regenerated cells were confirmed as the donor origin (Duchamp de Lageneste et al., 2018). These studies provided convincing evidence ruling out the contribution from circulation.

After the exclusion of circulation contribution, the regenerated cells were most likely of local origin. The next step is to determine whether they come from the periosteum, cortical bone, or bone marrow (BM). To solve this question, Colnot (2009) designed a series of bone graft transplantation experiments. Bone grafts from RosaLac-Z mice with or without periosteum were transplanted into wild-type mice. By tracing the Lac-Z-positive cells, it was concluded that repairing cells came from adjacent tissues; cells derived from the periosteum were always found on the periosteal surface; cells derived from the BM were always found within the marrow cavity; and cortical bone involvement was very limited. In the callus, cartilage cells are almost always derived from the periosteum, while osteoblasts are derived from both the periosteum and BM (Colnot, 2009; Wang et al., 2019). In a similar study, the authors stated that approximately 70% of the total regenerated cells came from the periosteum (Zhang et al., 2005). Although both the bone marrow and periosteum contribute to the formation of new bone, the quantity is different. Because the majority of bone callus tissue is located beneath the periosteum, it is no surprise to find that most of the regeneration cells come from the periosteum. As shown by Kojimoto et al. (1988), bone regeneration is highly dependent on the periosteum rather than on the bone marrow. When the periosteum was removed during DO, bone regeneration was markedly disturbed. However, in contrast, scraping of BM had no pronounced effect (Kojimoto et al., 1988). Although in cases when the periosteum were removed, the cells from adjacent skeletal muscle tissue could be involved in bone repair as a compensation (Julien et al., 2021; Liu et al., 2011). When the periosteum is in its position, its regeneration capacity is sufficient, and other alternative cellular sources are not required (Liu et al., 2011).

All of these studies demonstrated the strong regenerative capacity of the periosteum. Furthermore, the capacity is believed to come from P-SSCs. In this review, we summarized the current knowledge of periosteal SSCs for their origin, identity, properties, and special response to injuries.

## THE CONCEPT OF SKELETAL STEM CELLS

SSCs are a concept developed from mesenchymal stem cells (MSCs). The term MSCs was first introduced by Caplan (1991) to describe a population of cells in the bone marrow that possesses trilineage (adipogenic, osteogenic, and chondrogenic) differentiation capacity (Caplan, 1991; Dominici et al., 2006). MSCs have long been considered to be a pure cell population, and each cell has trilineage differentiation potential. However, MSCs actually have high heterogeneity, and every individual cell is not equal in its differentiation capacity (Viswanathan et al., 2019). Leptin receptor (LepR) is a widely accepted marker of adult MSCs. In their physiological state, BM-LepR + cells give rise to bone and adipose tissue (Zhou et al., 2014). In a recent study, LepR + cells were divided into two subgroups: one enriched for osteogenic genes and the other enriched for adipogenic genes at the transcription level (Tikhonova et al., 2019). Under adipogenic induction, only already transcribed adipogenic genes would respond; others remained relatively quiescent (Tikhonova et al., 2019). Both Gremlin1 (Worthley et al., 2015) and PTHrP (Mizuhashi et al., 2018) have labeled a subgroup of MSCs residing within the growth plates. These cells could be differentiated into chondrogenic, osteogenic, and adipogenic lineage cells *in vitro*. However, they only generated bone, cartilage, reticular stromal, and no adipose cells in lineage tracing experiments (Worthley et al., 2015) (Mizuhashi et al., 2018). The same results were obtained from ectopic transplantation experiments. Chan et al. (2015, 2018) isolated CD45−Ter-119−Tie2−AlphaV + Thy−6C3−CD105−CD200+ cells in mice (Chan et al., 2015) and PDPN + CD146-CD73 + CD164+ cells in human growth plates (Chan et al., 2018) and transplanted them into the renal capsule. Only bone, cartilage, and stroma but not adipose tissue were generated. These data support the existence of at least two subgroups of MSCs: one poised for adipogenic differentiation and the other poised for osteogenic differentiation. Although MSCs can undergo trilineage differentiation *in vitro*, this phenomenon is induced by exogenous stimuli. In the absence of exogenous stimulation, the situation is different *in vivo*. In fact, large areas of the chromatin landscape need to be reshaped as osteogenic MSCs are driven to adipogenic differentiation (Meyer et al., 2016; Rauch et al., 2019). Therefore, each MSC is likely to carry a genetic preprogram that suggests a restricted differentiation direction. In some situations, such as in a living organism without exogenous stimuli, they are more likely to behave in a preprogrammed manner. The seemingly trilineage differentiation potential of MSCs is more likely to aggregate potential compounds of distinct cell types.

By focusing on the differentiation potential, "SSCs" are proposed to define a group of cells that already carry an

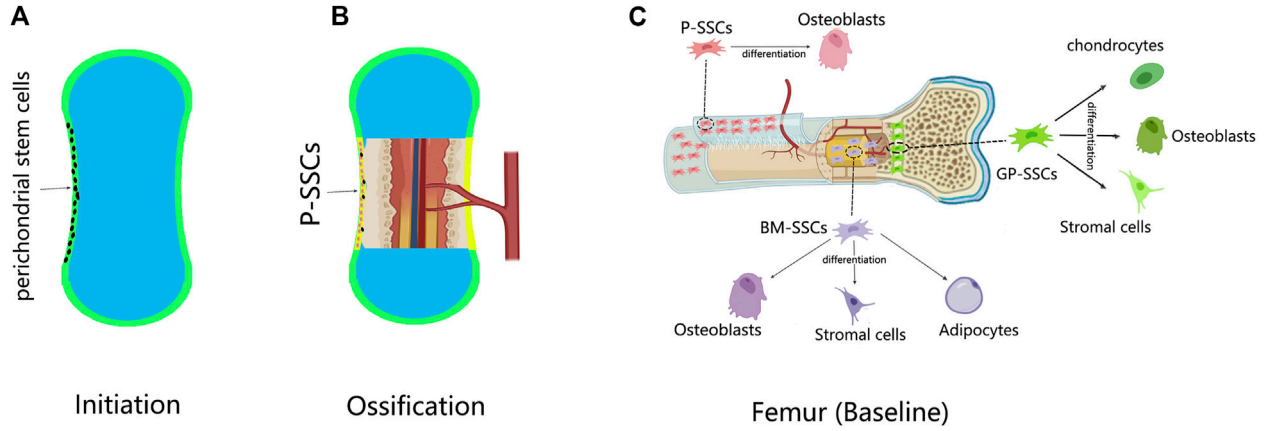


**FIGURE 1 |** The development of P-SSCs and their differentiation capacity. **(A)** In the limb development, a cartilage template is first established; the perichondrium contains undifferentiated mesenchymal cells. **(B)** Ossification begins when the blood vessel invades the center of the cartilage; the perichondrium is changed into periosteum. The perichondral mesenchymal cells give rise to P-SSCs. **(C)** Skeletal stem cells (SSCs) in different sites of the femur have distinct differentiation abilities. Compared with bone marrow-SSCs (BM-SSCs) and growth plate-SSCs (GP-SSCs) the periosteal SSCs (P-SSCs) can only give rise to osteoblasts at baseline.

osteogenic program that could differentiate into skeletal lineage (bone and cartilage) cells, excluding adipogenic lineages. However, the concept of "SSCs" is still in the development stage; "bone marrow "MSCs" and "SSCs" are currently used interchangeably. Only recently has the concept of SSCs been extrapolated from BM/growth plate (GP) to the periosteum.

## DEVELOPMENTAL ORIGIN OF THE PERIOSTEAL-SKELETAL STEM CELLS

Skeletons are formed by either intramembranous ossification or endochondral ossification. In intramembranous ossification, mesenchymal cells condense at the site of the future periosteum. Transcription factor 2 (Runx2) and osteogenic factor (Sp7) are then expressed in sequence, causing mesenchymal cells to differentiate directly into osteoblasts (Long, 2011). Undifferentiated mesenchymal cells form the periosteum, and some of them form P-SSCs (Ochareon and Herring, 2011) (**Figures 1A,B**).

In endochondral ossification, mesenchymal cells condense at the position of future bones (Akiyama et al., 2005). By expressing SRY-box 9 (Sox9), mesenchymal cells differentiate into chondrocytes to form cartilage templates (Akiyama et al., 2002; Akiyama et al., 2005; Long and Ornitz, 2013). The undifferentiated mesenchymal cells at the periphery form the perichondrium. At the same time, blood vessels invade the center of the cartilage template, forming the bone marrow cavity and transitioning from cartilage to bone tissue (Long and Ornitz, 2013). As ossification progresses, the perichondrium is reshaped into the periosteum (Kronenberg, 2007). During this time, the perichondral stem cells become P-SSCs. Therefore, whether P-SSCs are derived from local skeletal elements or from blood has not been elucidated until recently. Duchamp de Lageneste et al. (2018) transplanted unvascularized femur cartilage templates from Prx1-Cre and YFPfl/+ donors to the renal capsule of wild-type hosts. After 8 weeks, the cartilage templates developed into bone tissue, and the P-SSCs came from the donor (Duchamp de Lageneste et al., 2018). Lineage tracing experiments also provided evidence from another aspect. Hox11 is regionally expressed in the perichondrium of zeugopod limbs during the embryonic stage. Hox11-expressing cells persist in the periosteum continuously, giving rise to SSCs and osteoprogenitors from the embryonic to adult stage (Pineault et al., 2019). These experiments demonstrated the local origin of P-SSCs.

## FINDING A GOOD MARKER FOR PERIOSTEAL-SKELETAL STEM CELLS

P-SSCs and BM-SSCs are comparable to a great degree. Some markers, such as Nestin and LepR, used to identify BM-SSCs also label P-SSCs (Gao et al., 2019). BM, however, appears to be a much larger pool of cells (Méndez-Ferrer et al., 2010; Zhou et al., 2014). In one study, distinguishing between P-SSCs and BM-SSCs using these markers was difficult. A strategy of combined markers (CD45−Ter-119−Tie2−AlphaV + Thy−6C3−CD105−CD200+) provided by Chan et al. (2015) to identify GP-SSCs was also effective for P-SSCs (Chan et al., 2015; Ransom et al., 2018; Tournaire et al., 2020). However, the population in the periosteum contains approximately 7%–8% mature osteoblasts (Matthews et al., 2021). α-SMA is another well-studied marker that was originally a pericyte marker. Pericytes exhibit "stemness" in various tissues, and they are considered tissue-resident stem cells (Crisan et al., 2008; Wong et al., 2015). α-SMA has been proven to label some (not all) SSCs in the periosteum (Diaz-Flores et al., 1992; Matthews et al., 2014). However, a recent study uncovered the heterogeneity of periosteal α-SMA+ cells by single-cell RNA-seq (Matthews et al., 2021). The periosteal α-SMA+ cell population comprises three clusters: P-SSCs, fibroblasts, and perivascular cells (Matthews et al., 2021). Therefore, α-SMA

alone is not appropriate for serving as a P-SSC marker. MX1 is a BM-SSC marker with some limitations. Mx1-Cre is broadly expressed in other lineages but is not expressed under normal circumstances (Park et al., 2012). Ortinau et al. (2019) combined these two strategies and successfully identified MX1 + α-SMA+ cells as P-SSCs.

Specifically, this combination only labeled P-SSCs, and no double-positive cells were seen in the BM (Ortinau et al., 2019). Prrx1 is a broad skeletal mesenchymal cell marker (ten Berge et al., 1998; Peterson et al., 2005) that is expressed in both the endosteum and periosteum in the early postnatal period. With increasing age, Prrx1 expression is gradually restricted in the periosteum to mark P-SSCs (Esposito et al., 2020). Another marker is the cysteine protease cathepsin K (CTSK). Historically, CTSK has been used as a marker of osteoclasts; however, this application is site specific. In the BM, CTSK-lineage cells were tartrate-resistant acid phosphatase (TRAP) positive, indicating osteoclasts; however, the CTSK-lineage cells in the periosteum were TRAP negative. The periosteal CTSK-lineage cells include three clusters: P-SSCs (CD200+ CD105−), periosteal progenitor 1 (PP1) (CD200−CD105−), and periosteal progenitor 2 (PP2) (CD105 + $CD200_{variable}$). Among them, P-SSCs are the most stem-like cells, and they are the precursors of PP1 and PP2 (Debnath et al., 2018). Ideally, the markers should be able to separate P-SSCs from BM-SSCs, thus making α-SMA + MX1, Prrx1, and CTSK good candidates.

Characterization of P-SSCs by gene expression analysis revealed that the α-SMA + MX1+ P-SSCs could highly express CD105, CD140a, Cxcl12, LepR, and Grem1 (Ortinau et al., 2019). Prrx1-lineage P-SSCs overexpressed PDGFRα, Grem1, Cxcl12, Nestin, and NG2 but not LepR (Duchamp de Lageneste et al., 2018). The CTSK-lineage P-SSC population contains Gremlin1+ and Nestin+ subsets but not LepR, CD140a, or CD146 (Debnath et al., 2018).

In conclusion, the markers that identify BM-SSCs may also identify P-SSCs. To date, α-SMA + MX1, Prrx1, and CTSK are P-SSC-specific markers, and there are overlapping subsets among them.

## THE STEMNESS OF PERIOSTEAL-SKELETAL STEM CELLS

"Stemness" means the ability to proliferate, self-renew, and differentiate. Traditionally, the colony-forming ability on plastic dishes is referred to as "proliferative/self-renewal," and the multilineage differentiation ability in certain induction media is called "multipotency." However, some authors have noted the shortcomings of these definitions. Colony-forming assays or expansion cultures demonstrate not self-renewal but rather proliferation capacity (Bianco and Robey, 2015). *In vitro* differentiation assays proved the plasticity rather than the differentiation capacity. To eliminate the effect of exogenous factors, Bianco and Robey (2015) suggested that the differentiation capacity should be assessed by heterotopic transplantation of non-doctored and non-induced cultures. In this review, we evaluated the stemness of SSCs mainly by their behaviors *in vivo*.

The self-renewal capacity of Mx1+αSMA + P-SSCs was evaluated by serial transplantation experiments. The donor Mx1+αSMA + P-SSCs were sorted and transplanted into calvarial injury sites of hosts with Matrigel. Four weeks later, the Mx1 + αSMA + P-SSCs repopulated and generated new bone. Following the second transplantation, the retransplanted Mx1 + αSMA + P-SSCs also maintained repopulation and differentiation capacity (Ortinau et al., 2019). Similarly, the self-renewal capacity of CTSK-lineage P-SSCs was analyzed by two successive rounds of heterotopic transplantation. Donor CTSK-lineage P-SSCs were transplanted into the mammary fat pad and kidney capsule of female hosts. In both rounds, the CTSK-lineage P-SSCs self-renewed and rebuilt the P-SSC pool. The CTSK-lineage P-SSCs maintained their immunophenotype and differentiation capacity after the last transplantation (Debnath et al., 2018).

For the differentiation capacity, the same conclusion was drawn from different SSC lineage experiments. Nestin-lineage (Tournaire et al., 2020), CTSK-lineage (Debnath et al., 2018), and Mx1 + αSMA + (Ortinau et al., 2019) P-SSCs only generated bone without cartilage or stroma in transplantation. This observation coincided with the intramembranous ossification function mediated by the periosteum (**Figure 1C**).

In conclusion, P-SSCs are true tissue-resident stem cells with the capacity to proliferate, self-renew, and differentiate into osteolineage cells.

## PERIOSTEAL-SKELETAL STEM CELLS VS. BONE MARROW-SKELETAL STEM CELLS

Considering how closely the BM is related to the periosteum, it is interesting to compare them. *In vitro*, P-SSCs grew faster, and secondary colony formation efficiency was higher at the same density of P-SSCs and BM-SSCs (Duchamp de Lageneste et al., 2018). In transplantation assays, BM-SSCs displayed a typical endochondral ossification model generating bone, cartilage tissue, and hematopoietic stroma. P-SSCs represent an intramembranous ossification model, producing only bone tissue without cartilage and hematopoietic stroma (Debnath et al., 2018) with a higher migration capacity. In wound healing assays, the wound closure time of P-SSCs was only approximately half that of BM-SSCs (Debnath et al., 2018). *In vivo*, intravital imaging showed that the Mx1 + αSMA + P-SSCs moved toward the injury site immediately after wounding, whereas MX1 + BM-SSCs remained *in situ* (Ortinau et al., 2019). When P-SSCs and BM-SSCs were transplanted into the bone fracture site, the P-SSCs quickly penetrated into the center of the callus, while the BM-SSCs stayed at the periphery of the callus (Duchamp de Lageneste et al., 2018). At the gene expression level, P-SSCs were enriched in genes related to "stemness," "limb development," and "extracellular matrix (ECM)," while BM-SSCs were enriched in "downregulation of stemness," "bone resorption," "immune cells," and "hematopoietic stem cells" (Duchamp de Lageneste et al., 2018). The gene expression patterns coincide with their

distinct microenvironment. The BM is an immunological and hematopoietic organ; preserving immunological homeostasis and supporting hemopoiesis are also major functions of BM-SSCs (Méndez-Ferrer et al., 2010; Isern et al., 2014). This is why genes associated with hematopoiesis and immune response are enriched in BM-SSCs but not in P-SSCs. However, when we focus specifically on "stemness," P-SSCs appear to be the better choice.

In conclusion, compared with BM-SSCs, P-SSCs have higher growth and migration potential, but their differentiation capacity is more restricted in osteolineages.

## THE CONTRIBUTION OF PERIOSTEAL-SKELETAL STEM CELLS TO BONE REPAIR

As previously mentioned, P-SSCs are considered to be a major contributor to bone regeneration. In Prrx1-Cre, after activated recombination of mTmG mice 3 days after fracture (Esposito et al., 2020), all newly regenerated cells in the callus were derived from Prrx1+ cells (Duchamp de Lageneste et al., 2018).

Conditional ablation of Prrx1+ cells in Prx1-Cre$^{ER}$ DTA mice led to deficient healing with much less bone and cartilage content (Esposito et al., 2020). Similarly, MX1 + αSMA + P-SSCs contributed to approximately 20% of chondrocytes and 80% of new osteoblasts in the fracture callus (Ortinau et al., 2019). Conditional ablation of periosteal MX1+ cells led to significantly delayed healing and osteoblast reduction (Ortinau et al., 2019). Ablation of αSMA + cells either at the beginning or throughout the first 8 days in fracture healing led to reduced callus size formation and delayed mineralization (Matthews et al., 2021). By conditionally deleting OSX, a key osteogenic factor in the CTSK-Cre (Nakashima et al., 2002) CTSK + P-SSC osteogenic differentiation pathway was blocked. Osx$^{fl/fl}$ mice exhibited hypomineralization of the skull, uneven periosteal surfaces, and extensive linear intracortical pores (Debnath et al., 2018). Under injury conditions, these mice showed a markedly high risk of fracture non-union with defects in mineralization and an increased volume of cartilage in the callus (Debnath et al., 2018).

In conclusion, P-SSCs are the major sources of repairing cells in bone repair. The osteogenic differentiation capacity of these cells is critical to successful bone healing.

## THE SPECIAL RESPONSE OF PERIOSTEAL-SKELETAL STEM CELLS TO DIFFERENT BONE REGENERATION MODELS

In bone regeneration, endochondral and intramembranous ossification patterns occur simultaneously. Endochondral ossification predominated in unstable fracture models, but some direct bone formation and fibrochondrogenesis were also involved. Intramembranous ossification predominated in stable fracture, bone defect, and DO models (Runyan and Gabrick, 2017). In unstable fracture healing, cartilage scaffolds are first constructed and then gradually replaced with bone (Ferguson et al., 1999). Almost no cartilage is seen in intramembrane bone regeneration models (Jazrawi et al., 1998; Choi et al., 2002). P-SSCs respond differently to these patterns, but approximately 20% of available chromatin site alterations are common in fractures and DO models, including clusters related to general stress and inflammatory responses, such as HIF, VEGF, and IL signaling (Ransom et al., 2018). Here, we mainly focused on the modeled special responses of P-SSCs.

As we have discussed before, the periosteum not only mediates intramembranous ossification in physiological conditions but is also chondrogenic in fractures; this is a longstanding contradiction. In ectopic transplantation, Prrx1-, MX1 + αSMA-, and CTSK-labeled P-SSCs not only produced cartilage-less bone but also contributed approximately 100%, 20%, and 50% of the total chondroblasts in the fracture callus, respectively (Debnath et al., 2018; Duchamp de Lageneste et al., 2018; Ortinau et al., 2019).

Interestingly, the CTSK-lineage P-SSCs isolated from normal periosteum were osteoblastic only, but the P-SSCs isolated from the fractured periosteum were both osteoblastic and cartilaginous, and they could give rise to bone and cartilage in capsule transplantation (Debnath et al., 2018), indicating that a transition occurs in the P-SSCs. Genetically, P-SSCs upregulate cartilage formation-associated genes in fractures, such as Sox9 (Ransom et al., 2018), making the gene expression profiles similar to those of BM-SSCs. The fate shifting of P-SSCs in fractures has been observed, but its regulatory mechanism remains unclear. Some evidence points to the HIF and BMP pathways, but further investigation is required (Nakahara et al., 1990; Hanada et al., 2001; Ueno et al., 2001; Tsuji et al., 2006; Eyckmans et al., 2010; Chan et al., 2015).

The most significant difference between the fracture model and the DO model is that the DO model produces a larger bone tissue volume. The rate of bone growth during DO is equivalent to that of the fetus and is four–eight times that of adolescents (Steinbrech et al., 2000; Choi et al., 2002). Unlike the unstable fracture model that demonstrates the plasticity of P-SSCs, the DO model displays other sides of P-SSCs. Runx2 and Sox9 are key triggers of osteogenesis and chondroblast differentiation, with high chromatin accessible in fracture P-SSCs but not in DO (Ransom et al., 2018), indicating that P-SSCs are not in a poised differentiation state in DO but are ready to differentiate in fractures. The genes specifically enriched in P-SSCs during DO are associated with vascularization, adhesion, migration, and responses to mechanical stimulation (Ransom et al., 2018). Disruption of the mechanotransduction pathway FAK led to non-oriented migration of the P-SSCs and failure of DO (Ransom et al., 2018). These data indicated that the primary response of P-SSCs in DO had a close relationship with their migration within the periosteum. Human P-SSCs have been reported to express receptors from chemokine subfamilies CC, CXC, CX3C, and C, which respond to CCL2, CCL25, CXCL8, CXCL12, and CXCL13 (Stich et al., 2008). In mice, MX1 + αSMA + P-SSCs induced high expression of CCR5 and recruitment to the bone defect site (Ortinau et al., 2019). Blocking the

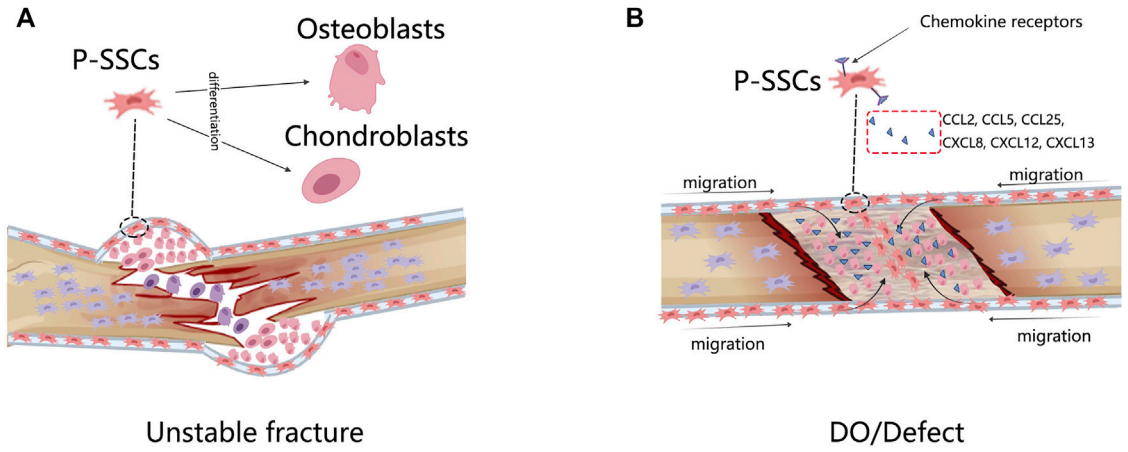


**FIGURE 2 |** The response of P-SSCs in different bone injury models. **(A)** In unstable fracture condition, P-SSCs acquire the capacity to differentiate into chondrocytes. **(B)** In DO or defected condition, P-SSCs showed high migratory capabilities. They are recruited into the bone defect site in response to special migration signals.

CCL5–CCR5 interaction severely interrupts the bone healing process (Ortinau et al., 2019) (**Figure 2**).

In conclusion, during unstable fracture healing, osteogenic unipotent P-SSCs become osteogenic and chondrogenic bipotent. The complete the migration capability of the P-SSCs is fundamental to successful DO.

## DISCUSSION

In this review, the research progress on P-SSC properties in recent years was summarized. P-SSCs are a population of cells with high proliferation, migration, and osteogenic potential that exhibit plasticity in fractures, but the mechanism regulating this cell fate transition has not yet been fully clarified. The high mobility of P-SSCs is critical to DO. Thoroughly understanding the properties of P-SSCs and their behavior in injury is the cornerstone for their potential clinical application.

Since the discovery of tissue-resident stem cells, they have been widely used in both fundamental and clinical research. BM-SSCs have shown plasticity *in vitro*, and subsequently, many research groups have adopted BM-SSC therapy with the hope that BM-SSCs can cure diseases by differentiating into local cell types. However, BM-SSCs rarely or never differentiate into cell types outside the skeletal lineage (Caplan, 2017). Within the skeletal lineage, when considering reconstruction of the cartilage, synovium-derived stem cells showed a higher chondrogenic potential than BM-SSCs (Sasaki et al., 2018). Cartilage regeneration using BM-SSCs requires genetic modification or induction (Chan et al., 2015), but perichondrium-derived stem cells could directly form cartilage (Kobayashi et al., 2011). Similarly, P-SSCs appear to be a better choice than BM-SSCs for bone tissue reconstruction, where we do not want to see too much cartilage. Tissue-resident stem cells carry tissue-specific memories. Therefore, we need to be very careful when trying to use SSCs as a therapy. Before transplantation, we need to determine whether cartilage or bone is needed. In other words, the best cells should be chosen for the best treatment.

## AUTHOR CONTRIBUTIONS

JP designed this study; LH and ZC collected the articles; NZ summarized the data; XL made the figures. All authors contributed to the article and approved the submitted version.

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## REFERENCES

Akiyama, H., Chaboissier, M.-C., Martin, J. F., Schedl, A., and de Crombrugghe, B. (2002). The Transcription Factor Sox9 Has Essential Roles in Successive Steps of the Chondrocyte Differentiation Pathway and Is Required for Expression of Sox5 and Sox6. *Genes Dev.* 16 (21), 2813–2828. doi:10.1101/gad.1017802

Akiyama, H., Kim, J.-E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., et al. (2005). Osteo-chondroprogenitor Cells Are Derived from Sox9 Expressing Precursors. *Proc. Natl. Acad. Sci.* 102 (41), 14665–14670. doi:10.1073/pnas.0504750102

Allen, M. R., Hock, J. M., and Burr, D. B. (2004). Periosteum: Biology, Regulation, and Response to Osteoporosis Therapies. *Bone* 35 (5), 1003–1012. doi:10.1016/j.bone.2004.07.014

Bianco, P., and Robey, P. G. (2015). Skeletal Stem Cells. *Development* 142 (6), 1023–1027. doi:10.1242/dev.102210

Buckwalter, J. A., and Cooper, R. R. (1987). Bone Structure and Function. *Instr. Course Lect* 36, 27–48.

Caplan, A. I. (1991). Mesenchymal Stem Cells. *J. Orthop. Res.* 9 (5), 641–650. doi:10.1002/jor.1100090504

Caplan, A. I. (2017). Mesenchymal Stem Cells: Time to Change the Name!. *Stem Cell Transl Med* 6 (6), 1445–1451. doi:10.1002/sctm.17-0051

Chan, C. K. F., Gulati, G. S., Sinha, R., Tompkins, J. V., Lopez, M., Carter, A. C., et al. (2018). Identification of the Human Skeletal Stem Cell. *Cell* 175 (1), 43–56. doi:10.1016/j.cell.2018.07.029

Chan, C. K. F., Seo, E. Y., Chen, J. Y., Lo, D., McArdle, A., Sinha, R., et al. (2015). Identification and Specification of the Mouse Skeletal Stem Cell. *Cell* 160 (1-2), 285–298. doi:10.1016/j.cell.2014.12.002

Chanavaz, M. (1995). The Periosteum: the "umbilical Cord" of Bone. Quantification of the Blood Supply of Cortical Bone of Periosteal Origin. *Rev. Stomatol Chir Maxillofac.* 96 (4), 262–267.

Choi, I. H., Chung, C. Y., Cho, T. J., and Yoo, W. J. (2002). Angiogenesis and Mineralization during Distraction Osteogenesis. *J. Korean Med. Sci.* 17 (4), 435–447. doi:10.3346/jkms.2002.17.4.435

Colnot, C. (2009). Skeletal Cell Fate Decisions within Periosteum and Bone Marrow during Bone Regeneration. *J. Bone Mineral Res.* 24 (2), 274–282. doi:10.1359/jbmr.081003

Crisan, M., Yap, S., Casteilla, L., Chen, C.-W., Corselli, M., Park, T. S., et al. (2008). A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. *Cell Stem Cell* 3 (3), 301–313. doi:10.1016/j.stem.2008.07.003

Debnath, S., Yallowitz, A. R., McCormick, J., Lalani, S., Zhang, T., Xu, R., et al. (2018). Discovery of a Periosteal Stem Cell Mediating Intramembranous Bone Formation. *Nature* 562 (7725), 133–139. doi:10.1038/s41586-018-0554-8

Diaz-Flores, L., Gutierrez, R., Lopez-Alonso, A., Gonzalez, R., and Varela, H. (1992). Pericytes as a Supplementary Source of Osteoblasts in Periosteal Osteogenesis. *Clin. Orthopaedics Relat. Res.* 275, 280–286. doi:10.1097/00003086-199202000-00042

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S., et al. (2006). Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy Position Statement. *Cytotherapy* 8 (4), 315–317. doi:10.1080/14653240600855905

Duchamp de Lageneste, O., Julien, A., Abou-Khalil, R., Frangi, G., Carvalho, C., Cagnard, N., et al. (2018). Periosteum Contains Skeletal Stem Cells with High Bone Regenerative Potential Controlled by Periostin. *Nat. Commun.* 9 (1), 773. doi:10.1038/s41467-018-03124-z

Dwek, J. R. (2010). The Periosteum: what Is it, where Is it, and what Mimics it in its Absence? *Skeletal Radiol.* 39 (4), 319–323. doi:10.1007/s00256-009-0849-9

Esposito, A., Wang, L., Li, T., Miranda, M., and Spagnoli, A. (2020). Role of Prx1-Expressing Skeletal Cells and Prx1-Expression in Fracture Repair. *Bone* 139, 115521. doi:10.1016/j.bone.2020.115521

Eyckmans, J., Roberts, S. J., Schrooten, J., and Luyten, F. P. (2010). A Clinically Relevant Model of Osteoinduction: a Process Requiring Calcium Phosphate and BMP/Wnt Signalling. *J. Cel Mol Med* 14 (6B), 1845–1856. doi:10.1111/j.1582-4934.2009.00807.x

Ferguson, C., Alpern, E., Miclau, T., and Helms, J. A. (1999). Does Adult Fracture Repair Recapitulate Embryonic Skeletal Formation? *Mech. Dev.* 87 (1-2), 57–66. doi:10.1016/s0925-4773(99)00142-2

Ferretti, C., and Mattioli-Belmonte, M. (2014). Periosteum Derived Stem Cells for Regenerative Medicine Proposals: Boosting Current Knowledge. *Wjsc* 6 (3), 266–277. doi:10.4252/wjsc.v6.i3.266

Gallardo-Calero, I., Barrera-Ochoa, S., Manzanares, M. C., Sallent, A., Vicente, M., López-Fernández, A., et al. (2019). Vascularized Periosteal Flaps Accelerate Osteointegration and Revascularization of Allografts in Rats. *Clin. Orthop. Relat. Res.* 477 (4), 741–755. doi:10.1097/CORR.0000000000000400

Gao, B., Deng, R., Chai, Y., Chen, H., Hu, B., Wang, X., et al. (2019). Macrophage-lineage TRAP+ Cells Recruit Periosteum-Derived Cells for Periosteal Osteogenesis and Regeneration. *J. Clin. Invest.* 129 (6), 2578–2594. doi:10.1172/JCI98857

Hanada, K., Solchaga, L. A., Caplan, A. I., Hering, T. M., Goldberg, V. M., Yoo, J. U., et al. (2001). BMP-2 Induction and TGF-?1 Modulation of Rat Periosteal Cell Chondrogenesis. *J. Cel. Biochem.* 81 (2), 284–294. doi:10.1002/1097-4644(20010501)81:2<284:aid-jcb1043>3.0.co;2-d

Isern, J., García-García, A., Martín, A. M., Arranz, L., Martín-Pérez, D., Torroja, C., et al. (2014). The Neural Crest Is a Source of Mesenchymal Stem Cells with Specialized Hematopoietic Stem Cell Niche Function. *Elife* 3, e03696. doi:10.7554/eLife.03696

Jaloux, C., Bettex, Q., Levadoux, M., Cerlier, A., Iniesta, A., Legre, R., et al. (2020). Free Vascularized Medial Femoral Condyle Corticoperiosteal Flap with Non-vascularized Iliac Crest Graft for the Treatment of Recalcitrant Clavicle Non-union. *J. Plast. Reconstr. Aesthet. Surg.* 73 (7), 1232–1238. doi:10.1016/j.bjps.2020.03.018

Jazrawi, L. M., Majeska, R. J., Klein, M. L., Kagel, E., Stromberg, L., and Einhorn, T. A. (1998). Bone and Cartilage Formation in an Experimental Model of Distraction Osteogenesis. *J. Orthopaedic Trauma* 12 (2), 111–116. doi:10.1097/00005131-199802000-00008

Julien, A., Kanagalingam, A., Martínez-Sarrà, E., Megret, J., Luka, M., Ménager, M., et al. (2021). Direct Contribution of Skeletal Muscle Mesenchymal Progenitors to Bone Repair. *Nat. Commun.* 12 (1), 2860. doi:10.1038/s41467-021-22842-5

Kobayashi, S., Takebe, T., Inui, M., Iwai, S., Kan, H., Zheng, Y.-W., et al. (2011). Reconstruction of Human Elastic Cartilage by a CD44+ CD90+ Stem Cell in the Ear Perichondrium. *Proc. Natl. Acad. Sci.* 108 (35), 14479–14484. doi:10.1073/pnas.1109767108

Kojimoto, H., Yasui, N., Goto, T., Matsuda, S., and Shimomura, Y. (1988). Bone Lengthening in Rabbits by Callus Distraction. The Role of Periosteum and Endosteum. *The J. Bone Jt. Surg. Br. volume* 70-B (4), 543–549. doi:10.1302/0301-620X.70B4.3403595

Kronenberg, H. M. (2007). The Role of the Perichondrium in Fetal Bone Development. *Ann. New York Acad. Sci.* 1116, 59–64. doi:10.1196/annals.1402.059

Lapierre, F., Masquelet, A., Aesch, B., Romana, C., and Goga, D. (1991). Cranioplasties Using Free Femoral Osteo-Periostal Flaps. *Chirurgie* 117 (4), 293–297.

Liu, R., Birke, O., Morse, A., Peacock, L., Mikulec, K., Little, D. G., et al. (2011). Myogenic Progenitors Contribute to Open but Not Closed Fracture Repair. *BMC Musculoskelet. Disord.* 12, 288. doi:10.1186/1471-2474-12-288

Long, F. (2011). Building strong Bones: Molecular Regulation of the Osteoblast Lineage. *Nat. Rev. Mol. Cel Biol* 13 (1), 27–38. doi:10.1038/nrm3254

Long, F., and Ornitz, D. M. (2013). Development of the Endochondral Skeleton. *Cold Spring Harbor Perspect. Biol.* 5 (1), a008334. doi:10.1101/cshperspect.a008334

Mach, D. B., Rogers, S. D., Sabino, M. C., Luger, N. M., Schwei, M. J., Pomonis, J. D., et al. (2002). Origins of Skeletal Pain: Sensory and Sympathetic Innervation of the Mouse Femur. *Neuroscience* 113 (1), 155–166. doi:10.1016/s0306-4522(02)00165-3

Masquelet, A. C., Romana, M. C., Penteado, C. V., and Carlioz, H. (1988). Vascularized Periosteal Grafts. Anatomic Description, Experimental Study, Preliminary Report of Clinical Experience]. *Rev. Chir Orthop. Reparatrice Appar Mot.* 74 Suppl 2 (Suppl. 2), 240–243.

Matsuo, K., Ji, S., Miya, A., Yoda, M., Hamada, Y., Tanaka, T., et al. (2019). Innervation of the Tibial Epiphysis through the Intercondylar Foramen. *Bone* 120, 297–304. doi:10.1016/j.bone.2018.11.007

Matthews, B. G., Grcevic, D., Wang, L., Hagiwara, Y., Roguljic, H., Joshi, P., et al. (2014). Analysis of αSMA-Labeled Progenitor Cell Commitment Identifies Notch Signaling as an Important Pathway in Fracture Healing. *J. Bone Miner Res.* 29 (5), 1283–1294. doi:10.1002/jbmr.2140

Matthews, B. G., Novak, S., Sbrana, F. V., Funnell, J. L., Cao, Y., Buckels, E. J., et al. (2021). Heterogeneity of Murine Periosteum Progenitors Involved in Fracture Healing. *Elife* 10. doi:10.7554/eLife.58534

Méndez-Ferrer, S., Michurina, T. V., Ferraro, F., Mazloom, A. R., Macarthur, B. D., Lira, S. A., et al. (2010). Mesenchymal and Haematopoietic Stem Cells Form a Unique Bone Marrow Niche. *Nature* 466 (7308), 829–834. doi:10.1038/nature09262

Meyer, M. B., Benkusky, N. A., Sen, B., Rubin, J., and Pike, J. W. (2016). Epigenetic Plasticity Drives Adipogenic and Osteogenic Differentiation of Marrow-Derived Mesenchymal Stem Cells. *J. Biol. Chem.* 291 (34), 17829–17847. doi:10.1074/jbc.M116.736538

Mizuhashi, K., Ono, W., Matsushita, Y., Sakagami, N., Takahashi, A., Saunders, T. L., et al. (2018). Resting Zone of the Growth Plate Houses a Unique Class of Skeletal Stem Cells. *Nature* 563 (7730), 254–258. doi:10.1038/s41586-018-0662-5

Moore, E. R., Zhu, Y. X., Ryu, H. S., and Jacobs, C. R. (2018). Periosteal Progenitors Contribute to Load-Induced Bone Formation in Adult Mice and Require Primary Cilia to Sense Mechanical Stimulation. *Stem Cel Res Ther* 9 (1), 190. doi:10.1186/s13287-018-0930-1

Nakahara, H., Bruder, S. P., Goldberg, V. M., and Caplan, A. I. (1990). *In Vivo* osteochondrogenic Potential of Cultured Cells Derived from the Periosteum. *Clin. Orthopaedics Relat. Res.* 259, 223–232. doi:10.1097/00003086-199010000-00032

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., et al. (2002). The Novel Zinc finger-containing Transcription Factor Osterix Is Required for Osteoblast Differentiation and Bone Formation. *Cell* 108 (1), 17–29. doi:10.1016/s0092-8674(01)00622-5

Ochareon, P., and Herring, S. W. (2011). Cell Replication in Craniofacial Periosteum: Appositional vs. Resorptive Sites. *J. Anat.* 218 (3), 285–297. doi:10.1111/j.1469-7580.2010.01336.x

Olsen, B. R., Reginato, A. M., and Wang, W. (2000). Bone Development. *Annu. Rev. Cel Dev. Biol.* 16, 191–220. doi:10.1146/annurev.cellbio.16.1.191

Ortinau, L. C., Wang, H., Lei, K., Deveza, L., Jeong, Y., Hara, Y., et al. (2019). Identification of Functionally Distinct Mx1+αSMA+ Periosteal Skeletal Stem Cells. *Cell Stem Cell* 25 (6), 784–796. doi:10.1016/j.stem.2019.11.003

Park, D., Spencer, J. A., Koh, B. I., Kobayashi, T., Fujisaki, J., Clemens, T. L., et al. (2012). Endogenous Bone Marrow MSCs Are Dynamic, Fate-Restricted Participants in Bone Maintenance and Regeneration. *Cell Stem Cell* 10 (3), 259–272. doi:10.1016/j.stem.2012.02.003

Peterson, R. E., Hoffman, S., and Kern, M. J. (2005). Opposing Roles of Two Isoforms of the Prx1 Homeobox Gene in Chondrogenesis. *Dev. Dyn.* 233 (3), 811–821. doi:10.1002/dvdy.20412

Pineault, K. M., Song, J. Y., Kozloff, K. M., Lucas, D., and Wellik, D. M. (2019). Hox11 Expressing Regional Skeletal Stem Cells Are Progenitors for Osteoblasts, Chondrocytes and Adipocytes throughout Life. *Nat. Commun.* 10 (1), 3168. doi:10.1038/s41467-019-11100-4

Rana, R. S., Wu, J. S., and Eisenberg, R. L. (2009). Periosteal Reaction. *Am. J. Roentgenology* 193 (4), W259–W272. doi:10.2214/AJR.09.3300

Ransom, R. C., Carter, A. C., Salhotra, A., Leavitt, T., Marecic, O., Murphy, M. P., et al. (2018). Mechanoresponsive Stem Cells Acquire Neural Crest Fate in Jaw Regeneration. *Nature* 563 (7732), 514–521. doi:10.1038/s41586-018-0650-9

Rauch, A., Haakonsson, A. K., Madsen, J. G. S., Larsen, M., Forss, I., Madsen, M. R., et al. (2019). Osteogenesis Depends on Commissioning of a Network of Stem Cell Transcription Factors that Act as Repressors of Adipogenesis. *Nat. Genet.* 51 (4), 716–727. doi:10.1038/s41588-019-0359-1

Runyan, C. M., and Gabrick, K. S. (2017). Biology of Bone Formation, Fracture Healing, and Distraction Osteogenesis. *J. Craniofac. Surg.* 28 (5), 1380–1389. doi:10.1097/SCS.0000000000003625

Sasaki, A., Mizuno, M., Ozeki, N., Katano, H., Otabe, K., Tsuji, K., et al. (2018). Canine Mesenchymal Stem Cells from Synovium Have a Higher Chondrogenic Potential Than Those from Infrapatellar Fat Pad, Adipose Tissue, and Bone Marrow. *PLoS One* 13 (8), e0202922. doi:10.1371/journal.pone.0202922

Squier, C. A., Ghoneim, S., and Kremenak, C. R. (1990). Ultrastructure of the Periosteum from Membrane Bone. *J. Anat.* 171, 233–239.

Steinbrech, D. S., Mehrara, B. J., Rowe, N. M., Dudziak, M. E., Luchs, J. S., Saadeh, P. B., et al. (2000). Gene Expression of TGF-??, TGF-?? Receptor, and Extracellular Matrix Proteins during Membranous Bone Healing in Rats. *Plast. Reconstr. Surg.* 105 (6), 2028–2038. doi:10.1097/00006534-200005000-00018

Stich, S., Loch, A., Leinhase, I., Neumann, K., Kaps, C., Sittinger, M., et al. (2008). Human Periosteum-Derived Progenitor Cells Express Distinct Chemokine Receptors and Migrate upon Stimulation with CCL2, CCL25, CXCL8, CXCL12, and CXCL13. *Eur. J. Cel Biol.* 87 (6), 365–376. doi:10.1016/j.ejcb.2008.03.009

ten Berge, D., Brouwer, A., Korving, J., Martin, J. F., and Meijlink, F. (1998). Prx1 and Prx2 in Skeletogenesis: Roles in the Craniofacial Region, Inner Ear and Limbs. *Development* 125 (19), 3831–3842. doi:10.1242/dev.125.19.3831

Tikhonova, A. N., Dolgalev, I., Hu, H., Sivaraj, K. K., Hoxha, E., Cuesta-Domínguez, Á., et al. (2019). The Bone Marrow Microenvironment at Single-Cell Resolution. *Nature* 569 (7755), 222–228. doi:10.1038/s41586-019-1104-8

Tournaire, G., Stegen, S., Giacomini, G., Stockmans, I., Moermans, K., Carmeliet, G., et al. (2020). Nestin-GFP Transgene Labels Skeletal Progenitors in the Periosteum. *Bone* 133, 115259. doi:10.1016/j.bone.2020.115259

Tsuji, K., Bandyopadhyay, A., Harfe, B. D., Cox, K., Kakar, S., Gerstenfeld, L., et al. (2006). BMP2 Activity, Although Dispensable for Bone Formation, Is Required for the Initiation of Fracture Healing. *Nat. Genet.* 38 (12), 1424–1429. doi:10.1038/ng1916

Uddströmer, L. (1978). The Osteogenic Capacity of Tubular and Membranous Bone Periosteum: A Qualitative and Quantitative Experimental Study in Growing Rabbits. *Scand. J. Plast. Reconstr. Surg.* 12 (3), 195–205. doi:10.3109/02844317809012995

Ueno, T., Kagawa, T., Mizukawa, N., Nakamura, H., Sugahara, T., and Yamamoto, T. (2001). Cellular Origin of Endochondral Ossification from Grafted Periosteum. *Anat. Rec.* 264 (4), 348–357. doi:10.1002/ar.10024

Viswanathan, S., Shi, Y., Galipeau, J., Krampera, M., Leblanc, K., Martin, I., et al. (2019). Mesenchymal Stem versus Stromal Cells: International Society for Cell & Gene Therapy (ISCT) Mesenchymal Stromal Cell Committee Position Statement on Nomenclature. *Cytotherapy* 21 (10), 1019–1024. doi:10.1016/j.jcyt.2019.08.002

Wang, Y., Chen, L., Kang, M., Ling, L., Tian, F., Won-Kim, S. H., et al. (2019). The Fracture Callus Is Formed by Progenitors of Different Skeletal Origins in a Site-Specific Manner. *JBMR Plus* 3 (9), e10193. doi:10.1002/jbm4.10193

Wong, S.-P., Rowley, J. E., Redpath, A. N., Tilman, J. D., Fellous, T. G., and Johnson, J. R. (2015). Pericytes, Mesenchymal Stem Cells and Their Contributions to Tissue Repair. *Pharmacol. Ther.* 151, 107–120. doi:10.1016/j.pharmthera.2015.03.006

Worthley, D. L., Churchill, M., Compton, J. T., Tailor, Y., Rao, M., Si, Y., et al. (2015). Gremlin 1 Identifies a Skeletal Stem Cell with Bone, Cartilage, and Reticular Stromal Potential. *Cell* 160 (1-2), 269–284. doi:10.1016/j.cell.2014.11.042

Yachouh, J., Breton, P., Roux, J.-P., and Goudot, P. (2010). Osteogenic Capacity of Vascularised Periosteum: an Experimental Study on Mandibular Irradiated Bone in Rabbits. *J. Plast. Reconstr. Aesthet. Surg.* 63 (12), 2160–2167. doi:10.1016/j.bjps.2010.01.015

Yiannakopoulos, C. K., Kanellopoulos, A. D., Trovas, G. P., Dontas, I. A., and Lyritis, G. P. (2007). The Biomechanical Capacity of the Periosteum in Intact Long Bones. *Arch. Orthop. Trauma Surg.* 128 (1), 117–120. doi:10.1007/s00402-007-0433-5

Zhang, X., Xie, C., Lin, A. S., Ito, H., Awad, H., Lieberman, J. R., et al. (2005). Periosteal Progenitor Cell Fate in Segmental Cortical Bone Graft Transplantations: Implications for Functional Tissue Engineering. *J. Bone Miner Res.* 20 (12), 2124–2137. doi:10.1359/JBMR.050806

Zhou, B. O., Yue, R., Murphy, M. M., Peyer, J. G., and Morrison, S. J. (2014). Leptin-receptor-expressing Mesenchymal Stromal Cells Represent the Main Source of Bone Formed by Adult Bone Marrow. *Cell Stem Cell* 15 (2), 154–168. doi:10.1016/j.stem.2014.06.008

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REVIEW
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# Clinical Potential of Dental Pulp Stem Cells in Pulp Regeneration: Current Endodontic Progress and Future Perspectives

*Kyu Hwan Kwack*[1] *and Hyeon-Woo Lee*[2]*

[1]Department of Dentistry, Graduate School, Kyung Hee University, Seoul, South Korea, [2]Department of Pharmacology, School of Dentistry, Graduate School, Institute of Oral Biology, Kyung Hee University, Seoul, South Korea

Dental caries is a common disease that not only destroys the rigid structure of the teeth but also causes pulp necrosis in severe cases. Once pulp necrosis has occurred, the most common treatment is to remove the damaged pulp tissue, leading to a loss of tooth vitality and increased tooth fragility. Dental pulp stem cells (DPSCs) isolated from pulp tissue exhibit mesenchymal stem cell-like characteristics and are considered ideal candidates for regenerating damaged dental pulp tissue owing to their multipotency, high proliferation rate, and viability after cryopreservation. Importantly, DPSCs do not elicit an allogeneic immune response because they are non-immunogenic and exhibit potent immunosuppressive properties. Here, we provide an up-to-date review of the clinical applicability and potential of DPSCs, as well as emerging trends in the regeneration of damaged pulp tissue. In addition, we suggest the possibility of using DPSCs as a resource for allogeneic transplantation and provide a perspective for their clinical application in pulp regeneration.

**Keywords: regeneration medicine, dental pulp stem cells, dental pulp regeneration, immunomodulation, allogeneic transplantation, mesenchymal stem cells**

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***Correspondence:**
*Hyeon-Woo Lee*
*hyeonwoo@khu.ac.kr*

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## INTRODUCTION

Dental pulp is a tissue in the center part of the tooth, surrounded by dentin, and plays a crucial role in maintaining the vitality of teeth by supplying essential factors through the apical foramen. The neural network distributed in the pulp tissue through the apical foramen plays a role in protecting the teeth by recognizing harmful stimuli, and the blood vessels in the pulp tissue supply nutrients to the teeth and remove waste products. Dental pulp has high functional regenerative capacity as it is responsible for the maintenance as well as the repair of periodontal tissue in response to various types of damage. Dental pulp cells proliferate when periodontal tissue is damaged, migrate to the damaged site, then differentiate into odontoblasts to form reparative dentin (Tziafas et al., 2000; Dimitrova-Nakov et al., 2014). Dental caries is one of the most prevalent diseases worldwide and has maintained its prevalence and incidence over the past two decades (Kassebaum et al., 2015). According to the most recent epidemiological data, the overall prevalence of total caries among youth aged in the United States is 45.8% (Fleming and Afful, 2018). There are no symptoms in the initial stages of caries, and symptoms begin only when the carious lesion grows and progresses to the dentin (Selwitz et al., 2007). When dental caries progresses and an inflammatory reaction occurs in the dental pulp, pulp tissue ischemia with severe pain occurs. The current common clinical treatment involves

removing the damaged dental pulp tissue, disinfecting it, and then filling it with artificial fillings (Morotomi et al., 2019). Although initial root canal treatment (RCT) has a high success rate and a predictable prognosis after treatment, the possibility of reinfection still exists (Salehrabi and Rotstein, 2004; Torabinejad et al., 2007; de Chevigny et al., 2008). Even if the treatment is successful, the vitality of the pulp is lost, and the perception and immune function completely disappear, reducing the resistance to external stimuli and weakening the teeth (Miran et al., 2016). Moreover, when reinfection occurs and retreatment is performed, the success rate decreases, and repeated RCT makes the teeth more fragile and prone to cracking or even fracture of the roots (Van Nieuwenhuysen et al., 1994; Imura et al., 2007; Ng et al., 2008). To solve these problems, a tissue regeneration approach that replaces damaged pulp with healthy pulp is an ideal treatment option. Therefore, novel strategies for regenerating functional pulp are essential as pulp stem cells are emerging as promising candidates.

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into all types of cells derived from the three embryonic germ layers, including osteoblasts, chondroblasts, and adipocytes. MSCs have been found in various tissues, such as bone marrow, adipose tissue, and dental tissues (Pittenger et al., 1999; Bianco et al., 2001). Because of these characteristics, MSCs have been recognized as a promising source of stem cells in regenerative therapy. However, their application has limitations such as safety and accessibility issues; thus, MSC-like cells isolated from dental tissues have begun to attract attention. MSC-like cells isolated from dental tissues have the advantage of being easily accessible from extracted teeth or from periodontal tissues that come with the extracted teeth (Gronthos et al., 2000; Gronthos et al., 2002; Miura et al., 2003). In addition, they can differentiate into nerves and blood vessels, which are necessary structures constituting the pulp tissue, and can be cryopreserved to store cells. Importantly, they may not elicit an immune response in allogeneic transplantations because they are non-immunogenic and have strong immunosuppressive properties (Kwack et al., 2017). With these advantages, DPSCs are the most important source of stem cells for pulp tissue regeneration because they exist in the original dental pulp and are prone to repair the damaged dental pulp (Zhang et al., 2017). In this review, we focus on DPSCs and provide an up-to-date review of their potential, including their immunosuppressive properties. In addition, the latest clinical methods for the regeneration of pulp tissue and the clinical applicability/strategies of DPSCs have also been discussed.

## DENTAL CARIES/PULPITIS

Dental caries is a common disease worldwide; however, it shows no symptoms until certain degree of progression. Untreated caries in permanent teeth are the most prevalent worldwide, and the prevalence and incidence of dental caries has been steadily maintained (Kassebaum et al., 2015). Dental caries is caused by complex interactions between the dietary supply of fermentable carbohydrates, acid-producing bacteria, and many host factors, including saliva and teeth (Selwitz et al., 2007). As a result of these interactions, the bacteria form a biofilm and cause demineralization of the outermost hard part of the tooth, the enamel, by acidogenic byproducts of bacterial metabolism. As enamel demineralization continues, dentin is exposed to bacterial invasion, resulting in further demineralization and cavitation. If caries progresses untreated, they turn to deep caries that penetrate the entire thickness of the dentin with specific pulp exposure. When dental pulp cells are exposed to dental caries, they respond directly by expressing various chemokines and cytokines to promote cellular defense processes and attempt repair (Farges et al., 2015). Fibroblasts, the principal cells of the pulp, can secrete factors important for the recruitment of stem cells, and the recruited stem cells are directly involved in repair (Frozoni et al., 2012; Jeanneau et al., 2017). In addition, bone marrow fibrocytes play a role in early wound healing by migrating to the damaged pulp site (Yoshiba et al., 2018). Several progenitor cell populations, including DPSCs, migrate to injured pulp sites and differentiate into odontoblast-like cells during reparative dentinogenesis (Gronthos et al., 2002). To undergo such a pulpal repair process, low-grade inflammation must progress to stimulate the regenerative response (Cooper et al., 2010). If inflammation is not removed despite a series of repair processes, it will eventually lead to an inflammatory pulpal reaction, resulting in pulp necrosis and abscesses (Reeves and Stanley, 1966; Bergenholtz et al., 1982).

Direct pulp capping or pulpotomy is clinically performed when the pulp is exposed to inflammation to maintain the vitality of the pulp. Direct pulp capping with dental biomaterial was performed to protect the exposed vital pulp by promoting restorative dentin formation (**Figure 1A**). Therefore, the primary purpose of pulp capping is to protect the exposed pulp tissue from external stimuli, such as bacteria. Therefore, pulp capping does not involve any process to remove the pulp tissue. In contrast, pulpotomy involves the removal of an exposed area (2–3 mm) of the infected pulp tissue (European Society of Endodontology, 2006) (**Figure 1B**). Pulpotomy is a treatment method based on histological research findings that irreversible pulpitis causes inflammation of the coronal pulp, whereas inflammation of the pulp tissue in the root chamber is rare (Ricucci et al., 2014). Although pulp chamber pulpotomy is mainly used to allow apexogenesis in immature teeth, recent reports suggest that it may have promising long-term results in mature teeth as well (Simon et al., 2013; Taha and Khazali, 2017). However, if the bacterial infection and inflammatory reaction of the pulp continue without proper treatment, the pressure inside the pulp chamber increases significantly, resulting in ischemia of the pulp tissue with severe pain. To reduce the patient's pain by lowering the pressure inside the pulp chamber, pulpectomy was performed to remove all pulp tissue (**Figure 1C**).

## MESENCHYMAL STEM CELLS

MSCs, which have the potential for self-renewal and multiple differentiation, play an essential role in organ development and repair (Uccelli et al., 2008; Bianco et al., 2013; Frenette et al.,

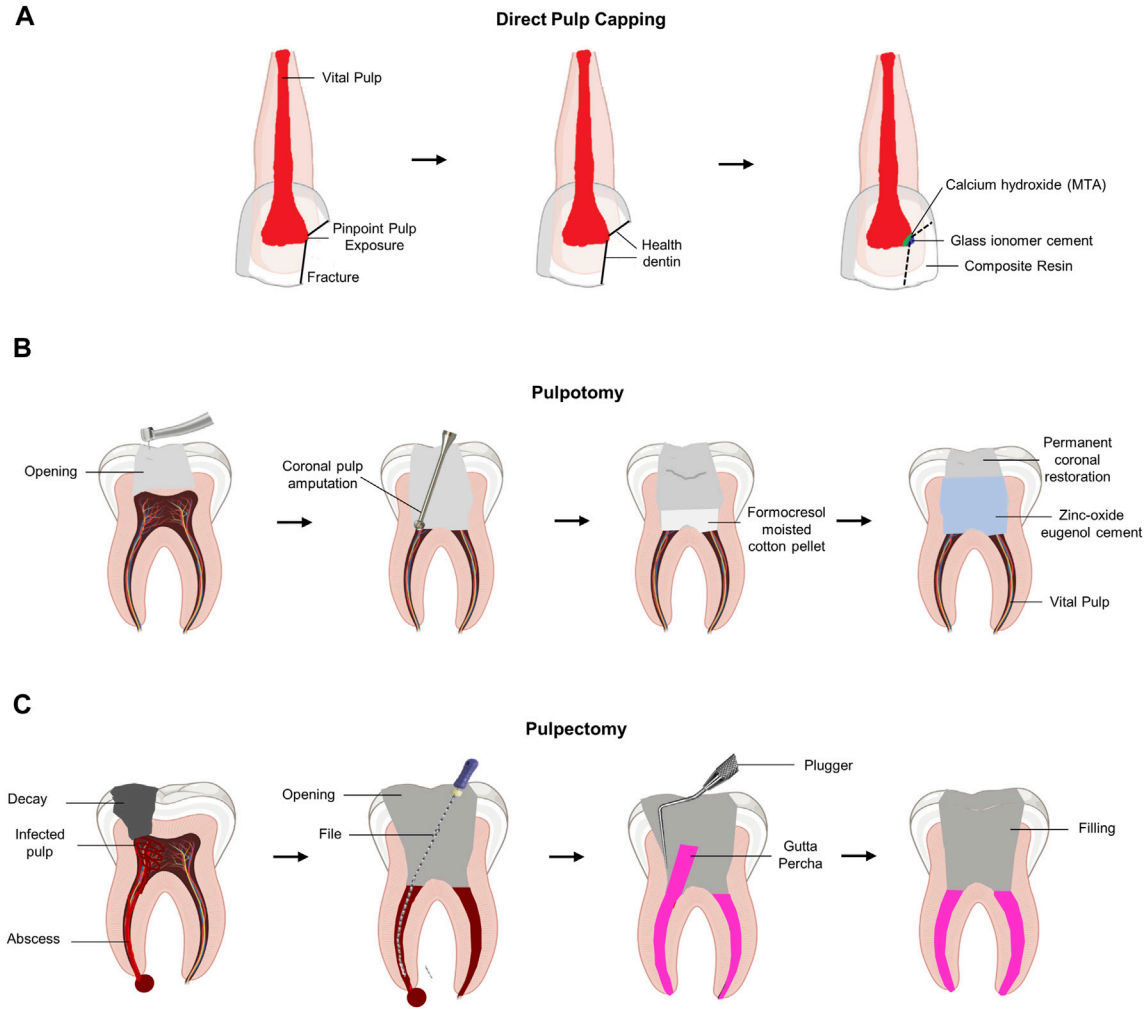


**FIGURE 1 |** Schematic diagram of the current concept of pulp therapy. **(A)** Direct pulp capping. Pulp capping is a method used to prevent necrosis of the dental pulp when it is slightly exposed to the pin-point. Direct pulp capping covers the exposed dental pulp with a base that protects the pulp and prevents infection to maintain dental pulp vitality. **(B)** Pulpotomy. Pulpotomy is a minimally invasive method that is clinically considered when there is pulpitis in the absence of root pathology. **(C)** Pulpectomy. Pulpectomy refers to a root canal treatment that removes irreversibly infected or necrotic pulp tissue.

2013). In various studies involving animal models and clinical trials, MSCs have received significant attention in regenerative medicine because of their tremendous potential to regenerate damaged tissues, including bones and teeth (Volponi et al., 2010; Daley, 2012; Grayson et al., 2015; Trounson and McDonald, 2015). MSCs have been proposed as an attractive cell source because of their ability to differentiate into osteoblasts or odontoblasts, to cryopreserve, and to modulate systemic immunity as well as avoid ethical disputes during the harvesting process, (Volponi et al., 2010; Daley, 2012; Grayson et al., 2015; Gao et al., 2017). Moreover, MSCs grow easily *in vitro* and are thus suitable for conducting various experiments in the field of regenerative medicine. MSCs can be identified by expressing cell surface markers such as CD73, CD90, and CD105 without expressing hematopoietic cell markers such as CD11b, CD34, and CD45 (Dominici et al., 2006). Human MSCs are multipotent cells isolated from various tissues, but the most common source tissues are bone marrow and adipose tissue (Haynesworth et al., 1992; Pittenger et al., 1999; Halvorsen et al., 2000; Zuk et al., 2001).

Bone marrow-derived MSCs have been extensively studied for bone regeneration because they strongly regulate bone homeostasis by regulating osteoblast differentiation and osteoclast activity (Frenette et al., 2013; Liu et al., 2014; Fernandes and Yang, 2016; Guo et al., 2018). With regard to bone homeostasis, cytotherapy or tissue engineering techniques have demonstrated therapeutic potential for the treatment of bone marrow-derived MSCs in osteopenia and bone defects (Shang et al., 2014; Liu S. et al., 2015; Sui et al., 2016; Sui et al., 2017). The iliac crest is mainly used to collect MSCs from bone marrow, and because bone marrow is renewable, it can be freely collected without ethical issues. However, harvesting the bone marrow is not readily accessible, as it requires conscious sedation and anesthesia, which requires monitoring by an anesthesiologist.

MSCs isolated from dental-related tissues exhibit typical MSC characteristics and have been found in various dental tissues, such as extracted teeth and adherent tissues (Gronthos et al., 2000; Gronthos et al., 2002; Miura et al., 2003; Seo et al., 2004). Unlike MSCs isolated from bone marrow, MSCs isolated from dental tissues are easily accessible because they can be isolated from wisdom teeth or healthy teeth that have been extracted for orthodontic purposes. It has been reported that the frequency of extraction of the four first premolars among orthodontic patients is as high as 8.9%–13.4% (Jackson et al., 2017). In addition, based on the dentist's value and empirical evidence, many asymptomatic third molars are extracted for prophylactic purposes prior to orthodontic treatment (Bastos Ado et al., 2016). Considering these points, MSCs isolated from dental-related tissues can be obtained more easily than MSCs isolated from bone marrow. The easily accessible dental-derived MSCs include periodontal ligament stem cells, stem cells from apical papilla, dental follicle cells, stem cells from human exfoliated deciduous teeth, and DPSCs.

## Periodontal Ligament Stem Cells

The periodontal ligament is a fibrous connective tissue that plays an important role in supporting teeth by anchoring them to the alveolar bone (Chen et al., 2012). Periodontal ligament stem cells isolated from the periodontal ligament are known to play a role in maintaining the function of periodontal tissue and regenerating the structure (Seo et al., 2004). *In vitro* experiments showed that periodontal ligament stem cells expressed cementoblast/osteoblast markers in culture and could be mineralized. In case of periodontal tissue defects, locally transplanted periodontal ligament stem cells migrate to and repair the defect, suggesting the possibility of periodontal tissue regeneration (Liu et al., 2008; Ding et al., 2010).

## Stem Cells from the Apical Papilla

The apical papilla tissue is located at the tip of the growing tooth root because it exists only during the development of the tooth root (Sonoyama et al., 2006; Huang et al., 2008; Sonoyama et al., 2008). The differentiation of stem cells from the apical papilla into odontoblasts and osteoblasts was confirmed *in vitro*, and the possibility of regeneration into cementum and periodontal ligament-like complexes was indicated *in vivo* (Han et al., 2010).

## Dental Follicle Cells

The dental follicle is a loose connective tissue derived from the ectomesenchyme that surrounds the dental papilla and the enamel of the developing tooth germ before eruption. Dental follicle cells, including progenitors of cementoblasts, periodontal ligaments, and osteoblasts, were found to differentiate into cementum *in vitro* (Kemoun et al., 2007; Yao et al., 2008) and *in vivo* in an experiment that used implants (Handa et al., 2002). In addition, dental follicle cells not only regenerate periodontal ligament-like tissues upon transplantation *in vivo* but also regenerate periodontal tissues through epithelial–mesenchymal interactions (Yokoi et al., 2007; Bai et al., 2011). Dental follicle cells are attracting attention in regenerative medicine because they maintain the characteristics of MSCs and form periodontal tissues even when sub-cultured more than other stem cells (Guo et al., 2012).

## Stem Cells from Human Exfoliated Deciduous Teeth

A cell population exhibiting the characteristics of MSCs was isolated from the pulp tissue of human exfoliated deciduous teeth and named "stem cells from human exfoliated deciduous teeth" (Miura et al., 2003). Stem cells from human exfoliated deciduous teeth can differentiate into osteoblasts *in vitro* (Su et al., 2016), and their differentiation into dentin-like tissues upon transplantation *in vivo* indicates that they have the potential for pulp regeneration (Miura et al., 2003; Shi et al., 2005; Cordeiro et al., 2008). Notably, stem cells from human exfoliated deciduous teeth showed a higher proliferation rate, osteogenic differentiation ability, and osteo-inductive potential compared to those of DPSCs and bone marrow-derived MSCs (Nakamura et al., 2009; Kunimatsu et al., 2018). Moreover, stem cells from human exfoliated deciduous teeth are considered an attractive cell source for bone and tooth regeneration because they can cryopreserve and maintain their differentiation potential even after cryopreservation (Ma et al., 2012).

## Dental Pulp Stem Cells

DPSCs are cells with MSC-like characteristics isolated from dental pulp that play a role in periodontal tissue repair and regeneration (Gronthos et al., 2000). DPSCs play an essential role in dentin repair and postnatal tooth homeostasis by differentiating into odontoblasts (Gronthos et al., 2002; Shi and Gronthos, 2003; Laino et al., 2005). Furthermore, because DPSCs are of neural origin, they can differentiate into glial cells and neurons and have also been shown to exhibit the ability to secrete neurotrophic factors that play a role in neurite outgrowth and neuroprotection (Arthur et al., 2008; Ratajczak et al., 2016) (**Figure 2**). Importantly, DPSCs possess a strong angiogenic capacity to generate capillary-like structures by secreting angiogenesis regulators under certain environmental conditions (Ratajczak et al., 2016) (**Figure 2**). Taken together, DPSCs with excellent neurodifferentiation and strong angiogenic potential, which are the most important factors for functional pulp regeneration, are the optimal cell source for dental pulp regeneration.

# DENTAL PULP STEM CELLS AS A SOURCE OF PULP REGENERATION

The pulp tissue receives blood vessels from the apical foramen to maintain vitality and is innervated to provide sensation to the teeth. Therefore, the strong angiogenic and neurogenic potential of MSCs is essential for the successful regeneration of pulp tissue. Therefore, the regenerated pulp tissue must 1) have a cell density and structure similar to those of the original pulp, 2) generate new dentin at a controlled rate similar to that of the original pulp, 3) have blood vessels formed and connected, and 4) innervated nerves (Fawzy El-Sayed et al., 2015). DPSCs have distinctive

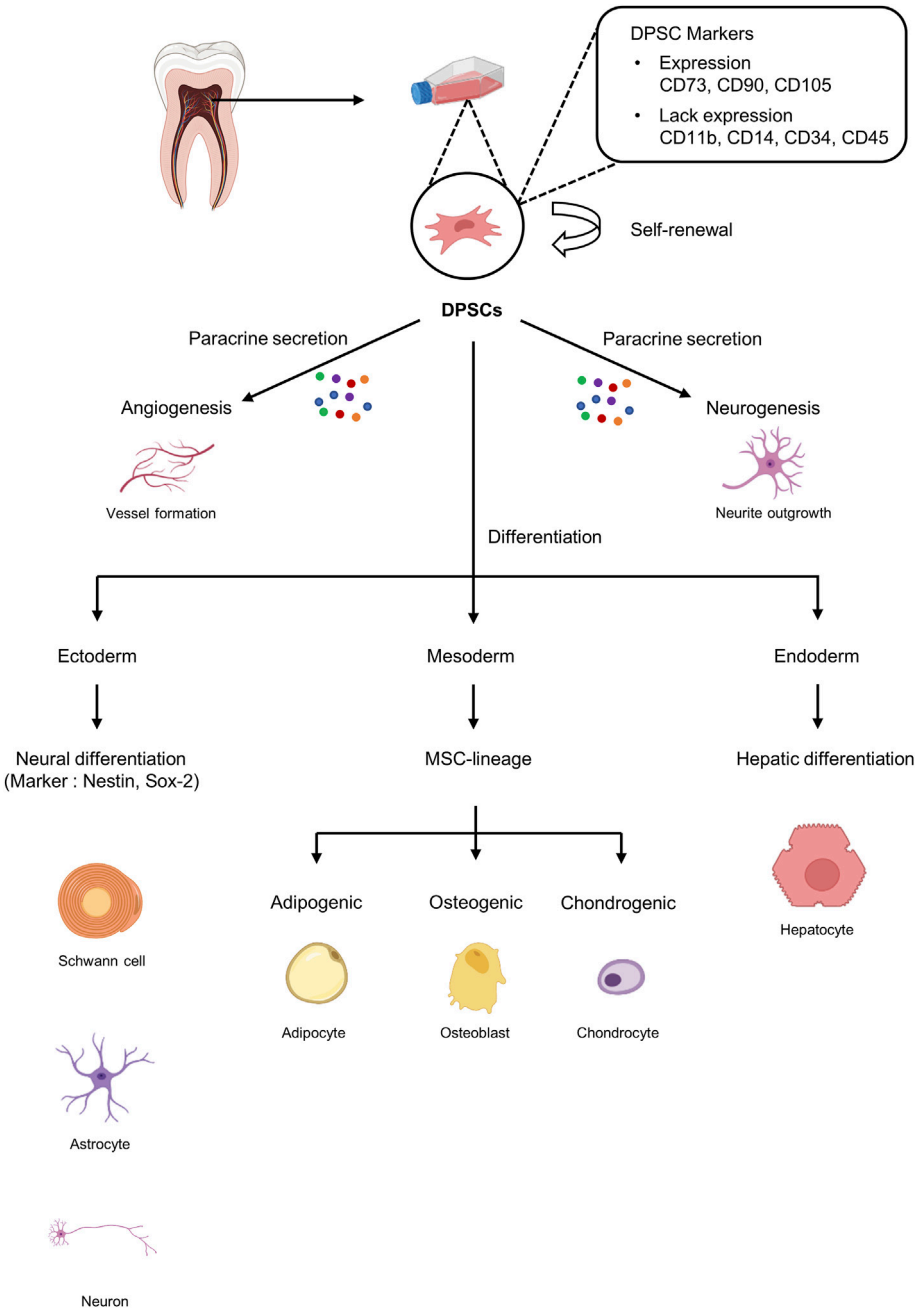


**FIGURE 2 |** Characteristics of dental pulp stem cells (DPSCs). DPSCs can be isolated from dental pulp tissue and express markers similar to those on mesenchymal stem cells (MSCs). DPSCs can undergo self-renewal and have the potential to differentiate into ectoderm, mesoderm, and endoderm.

neurovascular differentiation characteristics, suggesting that they may serve as the best candidates for pulp tissue regeneration (Gronthos et al., 2000). In addition, the pulp microenvironment maintains dynamic homeostasis, and these microenvironments must be closely mimicked during pulp regeneration. Thus, DPSCs surrounding the neurovascular bundle may be most suitable for pulp tissue regeneration (Shi and Gronthos, 2003; Zhao et al., 2014). Therefore, we focused on DPSCs and summarized their stemness, clinical application, immunomodulatory properties, and cryopreservation characteristics.

## Stemness

DPSCs are ectoderm-derived MSCs originating from the cranial neural crest cells. DPSCs have MSC-like properties, including fibroblast-like morphology and the ability to adhere to and proliferate on plastic surfaces, and exhibit MSC-like colony formation (Dominici et al., 2006; Martens et al., 2013). Similar

to MSCs, DPSCs express specific markers such as CD73, CD90, CD105, and STRO-1 but not hematopoietic markers such as CD11b and CD19 (Mattei et al., 2015). However, DPSCs are a heterogeneous population that also express a variety of other markers (**Figure 2**).

For successful tissue engineering, forming a rapid vascular network with the host circulatory system to supply the necessary oxygen and nutrients and remove waste products is essential (Jain et al., 2005). In relation to angiogenesis, DPSCs significantly upregulate pericyte markers such as NG2, platelet-derived growth factor receptor β (PDGFRβ), and α-smooth muscle actin (α-SMA) but do not express endothelial markers such as von Willebrand factor or CD31 (Janebodin et al., 2013). It has been reported that DPSCs support angiogenic and vasculogenic processes not only by secreting pro-angiogenic factors but also by direct differentiation into pericytes and endotheliocytes. Angiogenic potential has been established through pro-angiogenic factors like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), monocyte chemoattractant protein-1 (MCP-1), basic fibroblast growth factor (bFGF), and endothelin-1 (EDN1) secreted by DPSCs, and through these factors, DPSCs act as pericyte-like cells to stabilize blood vessels (Bronckaers et al., 2013; Janebodin et al., 2013).

The co-transplantation of endothelial progenitor cells and perivascular cells can form functional micro-vessels *in vivo* (Melero-Martin et al., 2007). Moreover, further administration of DPSCs can stabilize the pre-existing vasculature-like structure formed by human umbilical vein endothelial cells (HUVECs) and increase their longevity (Dissanayaka et al., 2012). When co-injected with HUVECs, DPSCs showed perivascular characteristics that contributed to angiogenesis (Nam et al., 2017). As such, it has been established that DPSCs are often closely associated with blood vessels, adopting the location and function of pericytes.

DPSCs derived from the cranial neural crest have neural properties, and in this regard, they are known to express nestin, a neural progenitor marker, and glial fibrillary acidic protein (GFAP), a glial marker (Davidson, 1994). DPSCs can differentiate into neural cells (Stevens et al., 2008) and glial cells (Gronthos et al., 2002) as well as neuronal nuclei (NeuN), neuron-specific markers that indicate neuronal differentiation capacity under neuronal induction conditions (Gronthos et al., 2002). DPSCs cultured in neuronal inductive media containing growth factors such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), are known to differentiate into neuron-like cells (Chang et al., 2014). Moreover, it was recently reported that optogenetic stimulation not only increases the vitality of DPSCs but also promotes differentiation to neuron-like cells (Niyazi et al., 2020).

In DPSCs, like other stem cells, side population (SP) cells characterized by a low level of Hoechst33342, a DNA-binding fluorescent dye, were found. Among the SP populations, the $CD31^{-}/CD146^{-}$ population is considered a promising population owing to the high expression of neurotrophic factors such as BDNF and nerve growth factor (NGF) and angiotrophic factors such as VEGF-A (Nakashima et al., 2009).

Therefore, DPSCs have cell characteristics suitable for angiogenesis and neurogenesis, which are essential for pulp regeneration.

## Immunomodulation Properties

Autologous DPSCs are considered a suitable source of cells for cell-based regenerative medicine but have limitations in that the uninfected teeth must be extracted and cryopreserved in a timely manner and used within the cryopreservation period. In contrast, using allogeneic cells has the advantage that when clinically applicable DPSCs are secured, a cell bank can be created and appropriately applied to patients in need at any time. However, allogeneic cell use can induce immune rejection by the host immune system due to a major histocompatibility complex (MHC) mismatch. DPSCs have effective and potent immunomodulatory functions to address immune rejection, suggesting their potential for regenerative medicine using allogeneic cells. Studies have primarily demonstrated the immunosuppressive properties of DPSCs through *in vitro* cell co-culture. Co-culture of stimulated T cells with DPSCs inhibits T-cell proliferation through the formation of regulatory T cells (Tregs), suggesting that Tregs may play a pivotal role in the immunosuppressive properties of DPSCs (Pierdomenico et al., 2005; Demircan et al., 2011; Hong et al., 2017) (**Figure 3**). Another study reported that DPSCs suppressed Th1 and Th2 subsets of $CD4^{+}$ T cells while increasing the proliferation of Treg and Th17 subsets (Ozdemir et al., 2016) (**Figure 3A**). In contrast, our group reported that Tregs are not directly related to the immunosuppressive properties of DPSCs. We reported that T cells are activated to secrete IFN-γ, which primes DPSCs to release TFG-β, thereby exhibiting immunosuppressive activity (Kwack et al., 2017) (**Figure 3B**). There was also a report that the Fas ligand expressed in DPSCs induces T-cell apoptosis through the Fas apoptotic pathway, resulting in the immunosuppressive property of DPSCs (Zhao et al., 2012). DPSCs participate in immune responses by interacting with macrophages and natural killer (NK) cells in addition to T cells. Transplanting DPSCs into unilateral hindlimb skeletal muscle showed that DPSCs could interact with macrophages to promote polarization toward the anti-inflammatory M2 phenotype (Omi et al., 2016). NK cells are considered important mediators in cell therapy because they efficiently lyse transplanted autologous and allogeneic MSCs (Spaggiari et al., 2006). However, DPSCs have been shown to increase resistance to NK cell lysis by overexpressing hypoxia-inducible factor 1 alpha (HIF-1α), thereby increasing the potential *in vivo* lifespan of transplanted DPSCs (Martinez et al., 2017).

DPSCs are known to modulate inflammatory factors, which downregulate the production of pro-inflammatory factors such as TNF-α, while upregulating the secretion of anti-inflammatory factors such as TGF-β (Demircan et al., 2011). Among them, TGF-β has been reported to be expressed in DPSCs as an immunosuppressive regulator and anti-inflammatory factor. It has been reported that DPSCs promote nerve repair and regeneration by releasing TGF-β in response to nerve damage and suppressing the acute immune response (Luo et al., 2018). These findings suggest

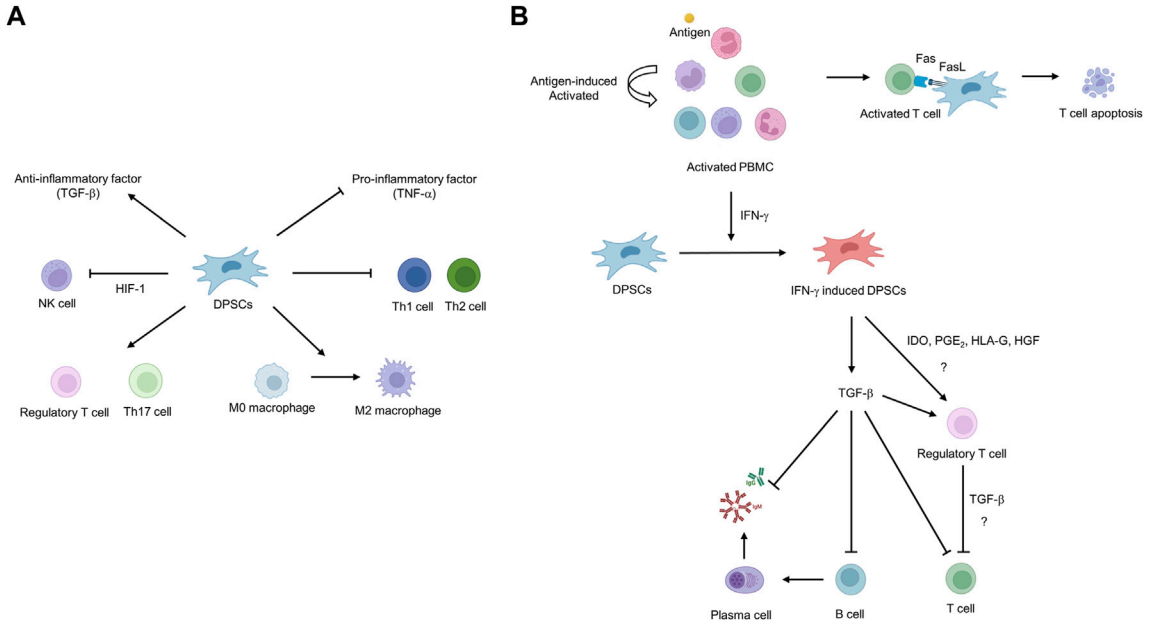


**FIGURE 3 |** Schematic diagram of the immunosuppressive potential of dental pulp stem cells (DPSCs). **(A)** DPSCs can inhibit the proliferation of natural killer (NK), T helper 1 (Th1), and T helper 2 (Th2) cells and the secretion of pro-inflammatory factors such as TNF-α. In addition, DPSCs induce the proliferation of regulatory T cells (Tregs)/T helper 17 (Th17) cells and differentiation toward macrophage M2 phenotype and secrete anti-inflammatory factors such as TGF-β. **(B)** Fas ligand expressed in DPSCs induces T-cell apoptosis through Fas apoptotic pathway. IFN-γ secreted from hyper-activated T cells primes DPSCs to secrete TGF-β, resulting in immunosuppressive ability.

that DPSCs may be a suitable source for allogeneic transplantation, as they not only exhibit immunomodulatory properties by regulating immune cell proliferation and cytokine production but are also involved in regulating inflammation.

However, activating immune cells *in vitro* is an artificial process and has a limitation in that it can hardly represent complex immune responses that are actually generated *in vivo*. The immunomodulatory properties of DPSCs are strongly influenced by the surrounding microenvironment and are generally not observed in steady-state quiescent DPSCs. MSCs are primed by inflammatory cytokines such as TNF-α and IFN-γ, which are released by activated immune cells. Primed MSCs, in turn, greatly enhance their immunosuppressive potential. Likewise, our group emphasized the importance of the surrounding microenvironment by demonstrating that the immunosuppressive effect disappeared when DPSCs were incubated with IFN-γ antibody to neutralize IFN-γ (Kwack et al., 2017). Further research is needed to understand the exact mechanism of the immunosuppression of DPSCs that occurs *in vivo*. However, according to a recent preclinical study of allogeneic transplantation of DPSCs after pulpectomy of canine incisors, the absence of side effects following transplantation of allogeneic mismatched DPSCs suggests that it exhibits immunosuppressive ability even *in vivo* (Iohara et al., 2018). Moreover, a recent report that the immunomodulatory effect of undifferentiated DPSCs is maintained during osteogenic differentiation supports the strong immunomodulatory ability of DPSCs (Hossein-Khannazer et al., 2019).

## Cryopreservation

Although DPSCs have regenerative activity for clinical applications, it is known that DPSCs isolated from the teeth of elderly patients or patients with systemic diseases such as systemic lupus erythematosus, rheumatoid arthritis, or diabetes have reduced bioactivity (Zhang J. et al., 2015). In particular, in elderly patients, not only does the pulp tissue shrink due to physiological secondary dentinogenesis and pathological tertiary dentinogenesis but also mineralization, such as pulpal stone, limits the acquisition of DPSCs, thus limiting its use. In addition, it is almost impossible to obtain and use the dental pulp of uninfected young patients on an as-needed basis. Therefore, cell banking is an essential technology for storing DPSCs in clinically appropriate conditions with minimal cell or tissue damage and applying them through immediate cell expansion when clinically needed (Woods et al., 2009). Cryopreservation is the process of maintaining cell viability by freezing and storing them at extremely low temperatures where biochemical reactions do not occur (Mullen and Critser, 2007). However, since cells are easily exposed to stressful conditions during cryopreservation, which leads to cryoinjury, research on how to prevent damage has been in progress for a long time. Cryoinjury can occur either through direct mechanical action due to the formation of ice crystals or by secondary effects due to changes in osmotic homeostasis (Pegg, 2015). To prevent such damage, cryoprotectants are used, of which the most widely used is dimethyl sulfoxide (DMSO), which penetrates through the cell cytoplasmic membrane and prevents the formation of ice crystal nuclei. However, because DMSO itself adversely affects cells

(causes cytotoxic effects), it is necessary to limit the concentration used for cell preservation. In general, it is recognized that a concentration of 10% or less is slightly toxic, and studies on the concentration of DMSO suitable for preservation of DPSCs are being conducted.

Importantly, for the application of DPSCs in regenerative medicine, cryopreservation should not affect their stemness features and multipotency. Several studies have shown that DPSCs can be cryopreserved while retaining their stem cell properties (Zhang et al., 2006; Gioventu et al., 2012). A recent study showed that DPSCs did not impair viability, proliferation, stemness, or differentiation capacity after cryopreservation at −80°C for 1 year (Pilbauerova et al., 2021). Despite these advantages, DPSCs can be cryopreserved only when an appropriate number is obtained by isolating and culturing DPSCs from pulp tissue after tooth extraction. This method takes a long time for cryopreservation, resulting in excessive labor and other costs, and there is even a risk of potential contamination by microorganisms. Therefore, there are studies moving from cell-level cryopreservation to tissue-level cryopreservation. There was no significant change in cell proliferation rates, cell growth morphology, and stem cell characteristics when pulp tissue was stored in liquid nitrogen for more than 1 year and then cultured (Han et al., 2017). Another study reported that the time lapse for cellular outgrowth was significantly reduced when 5% DMSO was used for cryopreservation of pulp tissue compared to when 10% DMSO was used without affecting other conditions (Yan et al., 2020). Therefore, it may be better to choose tissue cryopreservation over cell cryopreservation in that it positively affects the vitality of cells by reducing the time until cryopreservation, reducing the possibility of contamination, and reducing the direct toxic effects of DMSO on cells. Pulp tissue has been used as a scaffold for dental pulp regeneration (Hu et al., 2017; Song et al., 2017; Matoug-Elwerfelli et al., 2020; Bakhtiar et al., 2021), and cryopreservation as a tissue can be a good method for preserving the scaffold. In addition to these characteristics, DPSCs themselves have immunosuppressive properties (Kwack et al., 2017), and long-term cryopreservation weakens their immunogenicity (Yokomise et al., 1996), suggesting the possibility of allogeneic pulp tissue transplantation.

## Therapeutic Potential of Dental Pulp Stem Cells Related to Neurovascular Properties

Owing to the angiogenic and neurogenic potential of DPSCs, they are being studied for the treatment of various systemic diseases. In a study showing the neurodifferentiation properties of DPSCs, ectopic implantation of pulp tissue into the anterior chamber of rats resulted in innervation and upregulation of catecholaminergic nerve fiber density in the iris (Nosrat et al., 2001). In the same study, implantation of pulp tissue into hemisected spinal cords showed an increase in the number of surviving motor neurons, indicating that this effect is orchestrated by dental pulp-derived neurotrophic factors that functioned by rescuing motoneurons. Dental pulp-derived neurotrophic factors have been reported to have neuroprotective effects in Parkinson's disease by protecting dopaminergic neurons from MPP+ or rotenone toxicity *in vitro* (Nesti et al., 2011; Gnanasegaran et al., 2017). Moreover, DPSCs have been shown to have neurotrophic effects in Alzheimer's disease and Parkinson's disease (Apel et al., 2009; Martens et al., 2013; Zhang X.-M. et al., 2021). In particular, human dental pulp cells express a neuronal phenotype and produce neurotrophic factors such as NGF, GDNF, BDNF, and bone morphogenetic protein (BMP)-2, suggesting that they may be potential candidates for cell-based therapy.

The angiogenic potential of DPSCs has also been studied in several disease models. Functional revascularization was induced by transplantation of the $CD31^{-}$ $CD146^{-}$ SP of DPSCs isolated from porcine pulp tissue into the mouse hindlimb ischemia site (Iohara et al., 2008). The SP of DPSCs not only induces angiogenesis but also promotes the migration and differentiation of endogenous neural progenitors, thereby improving ischemic brain injury after middle cerebral artery occlusion (Sugiyama et al., 2011). In addition, the angiogenic potential of DPSCs can be determined from the study results that human DPSCs induce angiogenesis and alleviate infarction in rats with acute myocardial infarction (Gandia et al., 2008). The angiogenic potential of DPSCs was also shown in a model of muscular dystrophy, where DPSCs were engrafted into the host muscle, resulting in histological improvement by enhancing angiogenesis (Pisciotta et al., 2015). In dystrophic mouse models, human dental pulp pluripotent-like stem cells were engrafted into skeletal muscle and showed integration in muscle fibers and blood vessels by secreting several growth factors involved in angiogenesis (Martinez-Sarra et al., 2017).

# THE CLASSICAL CONCEPT OF PULP TISSUE ENGINEERING

Traditionally, infected dental pulp undergoes RCT in which all dental pulp is removed, and the pulp space is filled with artificial inorganic materials. However, teeth treated with the RCT method lose their vitality and become brittle, making them susceptible to postoperative fracture. Therefore, maintaining the vitality of dental pulp is an appropriate treatment to solve these problems. With the development of tissue engineering technology and regenerative medicine, efforts are being made to regenerate pulp tissue to maintain the vitality of teeth.

The three classical elements traditionally required in regenerative medicine are stem cells, scaffolds, and signaling molecules (growth factors), and the same concept has been used for dental pulp regeneration. Briefly, the concept is to isolate and culture stem cells *in vitro*, load them onto scaffolds, and apply them *in vivo* with signaling molecules that can help stem cells to properly differentiate.

The scaffold primarily serves as a tool to support stem cells, but it can also play a role in attracting cells or promoting

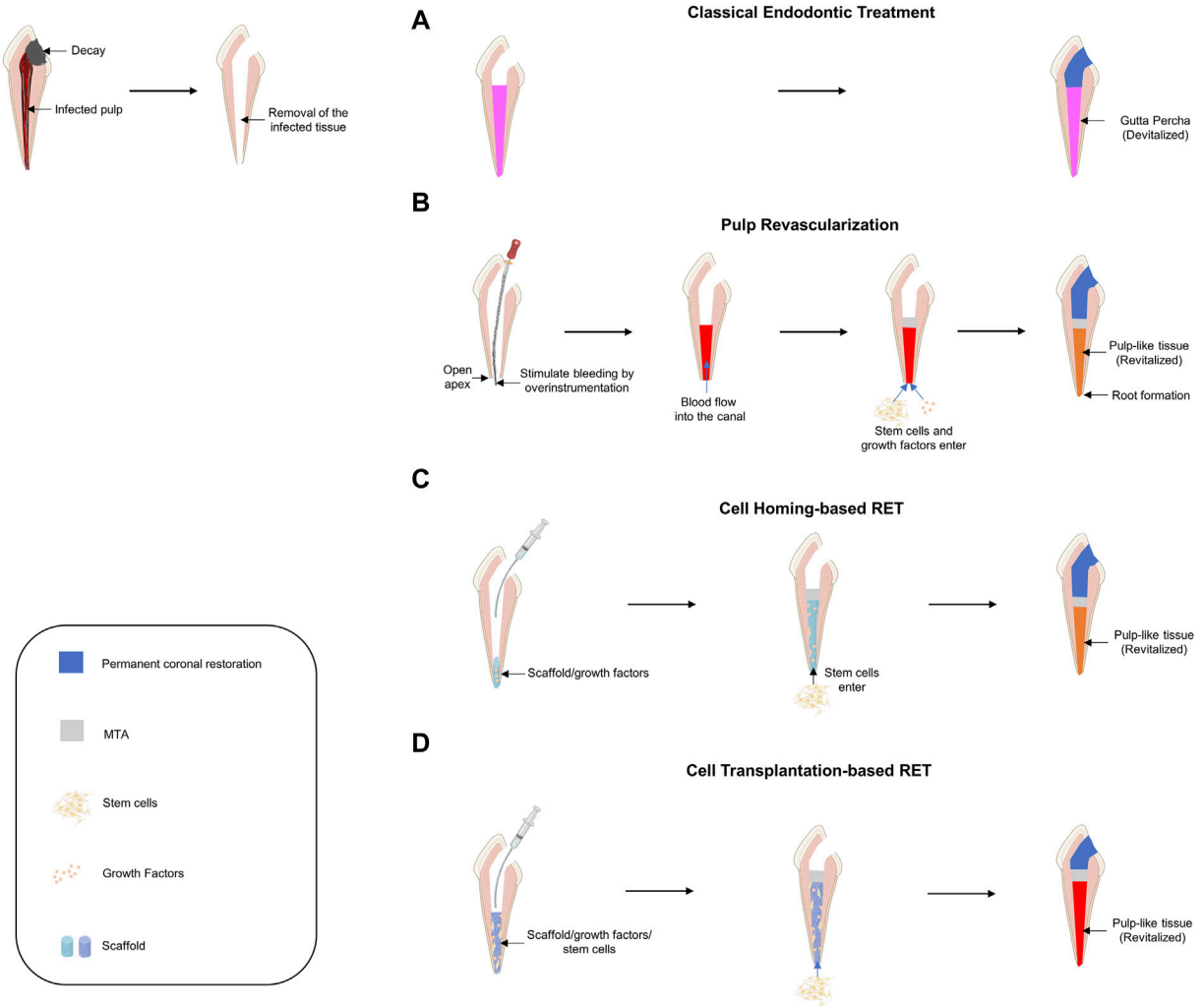


**FIGURE 4 |** Schematic diagram of current approaches for pulp tissue regeneration. **(A)** Classical endodontic treatment. **(B)** Pulp revascularization. **(C)** Cell-homing-based regenerative endodontic treatment (RET). **(D)** Cell-transplantation-based RET.

differentiation into specific cells by additionally loading growth factors or drugs (Brittberg et al., 1994). From a classical point of view, scaffolds are important for structural support, allow them to interact with their surrounding microenvironment, and can influence the signal pathway required for regeneration. To achieve this classical purpose, the scaffold must mechanically maintain its integrity, thereby supporting the adhesion and differentiation of stem cells to the implantation site. Additionally, the scaffold must mimic the original extracellular matrix of the tissue from which it is generated (Goldberg and Smith, 2004; Du and Moradian-Oldak, 2006).

Various exogenous growth factors have been demonstrated to enhance the migration, proliferation, and differentiation of DPSCs *in vitro*. Factors with excellent potential to induce the migration of DPSCs include bFGF (Suzuki et al., 2011; Takeuchi et al., 2015), stromal-derived factor-1 (SDF-1) (Suzuki et al., 2011; Yang et al., 2015) and granulocyte-colony stimulating factor (G-CSF) (Takeuchi et al., 2015). In addition, wnt3a (Hunter et al., 2015), G-CSF, and bFGF have been reported to promote the proliferation of DPSCs. Several factors are known to induce differentiation of DPSCs, and in particular, BMP-2 has been reported to induce their differentiation into odontoblasts (Oshima and Tsuji, 2014). Moreover, although BMP-7 had no significant effect on the recruitment of DPSCs, it induced mineralization of DPSCs (Suzuki et al., 2011), and TGF-β stimulated mineralization by differentiating DPSCs into odontoblast-like cells (Oshima and Tsuji, 2014). G-CSF was also a factor inducing dentinogenesis in DPSCs (Takeuchi et al., 2015); importantly, G-CSF is known to induce the migration, differentiation, and mineralization of DPSCs, as well as neurogenesis and angiogenesis, suggesting that it is an essential factor for pulp regeneration (Takeuchi et al., 2015). However, since the multiple actions of one factor may interfere with the sophisticated differentiation regulation for dental pulp regeneration, detailed mechanistic studies should be conducted.

## CURRENT APPROACHES FOR PULP TISSUE REGENERATION

If decayed teeth progress without proper treatment, pulp necrosis and abscesses accompanied by inflammatory pulp reactions occur. To save the infected tooth, an RCT was performed in which the entire infected pulp tissue was removed and disinfected, and the empty space was filled with artificial material (**Figure 4A**).

### Pulp Revascularization

Pulp revascularization is a procedure that regenerates the infected pulp tissue into pulp-like tissue by filling the root canal space with a blood clot after disinfection (**Figure 4B**). Because this procedure must induce a blood clot from the apical foramen, it can be used on immature teeth where the apical foramen has not yet closed. Attempts to regenerate pulp tissue by inducing blood clots into the root canal were first made in the 1960s (Ostby, 1961). If revascularization is successfully performed in immature teeth, root development of immature teeth can be completed; therefore, it has been actively studied in the field of traumatology (Skoglund et al., 1978; Galler, 2016). Many studies have been conducted, including the first case report on regenerative root canal treatment, and this treatment was adopted by the American Dental Association in 2011 and is now widely used in clinical practice (Iwaya et al., 2001; Banchs and Trope, 2004). Although pulp revascularization is similar to conventional root canal treatment in that it removes infectious agents, there are some differences in the basic concept. In RCT (or pulpectomy), to prevent re-infection, an aggressive disinfection process is performed, and artificial materials are filled in. In contrast, in pulp revascularization, mechanical debridement using an endodontic file is contraindicated to prevent damage to the root canal wall and induce the influx of stem cells located on the apical side to maintain the vitality of the tooth (Cvek, 1992; Iwaya et al., 2001). For this reason, in pulp revascularization, the use of intracanal medicaments along with the application of sufficient chemical disinfection is recommended instead of mechanical debridement. However, even if chemical disinfection is performed to protect the root canal wall, it is necessary to consider the balance between disinfection and cytotoxicity in stem cells. Thorough disinfection is important to prevent re-infection; however, an appropriate microenvironment for stem cell adhesion and differentiation is also needed for stem cell regeneration. Sodium hypochlorite, a representative chemical disinfectant, is known to be cytotoxic at a concentration of 3% or more and interferes with stem cell adhesion (Chang et al., 2001; Ring et al., 2008; Martin et al., 2014). Accordingly, the American Association of Endodontists recommends the use of a low concentration of sodium hypochlorite for pulp revascularization. Pulp revascularization faithfully follows the classical concept of tissue engineering. By inducing bleeding, MSCs from the apical side are delivered into the root canal (Lovelace et al., 2011), and the blood clot acts as a scaffold as well as a signaling molecule because of the presence of many growth factors (Shah et al., 2008; Nosrat et al., 2012). Reportedly, ethylenediaminetetraacetic acid (EDTA) can promote the differentiation of DPSCs into odontoblast-like cells by releasing various growth factors entrapped in dentin (Galler et al., 2011). Therefore, EDTA is recommended as the final irrigant. However, pulp revascularization has several limitations. As mentioned earlier, this method can only be used for immature teeth. Moreover, histological studies have shown that most tissues formed through pulp revascularization are not original pulp-like tissues but contain tissues such as periodontal, cementum, and bone-like tissues (Becerra et al., 2014). Therefore, further studies are needed to promote the formation of pulp-like tissue and to apply this method to mature teeth.

### Cell-Homing-Based Regenerative Endodontic Treatment

The basic concept of cell homing for dental regeneration is to achieve tissue regeneration through chemotaxis of host endogenous cells to damaged pulp tissue via signaling molecules, just as our body does for damaged tissue repair (Kim et al., 2010) (**Figure 4C**). Pulp revascularization and cell-homing-based RET is considered cell-free RET, as it is performed without exogenous cell transplantation. In pulp revascularization, blood acts not only as a scaffold but also as a source for signaling molecules, but cell homing can be applied to a scaffold that is advantageous for stem cell migration and proliferation and can load desired signaling molecules together. Because the cell-homing strategy is to create a suitable environment for the induction, differentiation, and proliferation of endogenous stem cells capable of regenerating pulp-dentin, it is important to identify endogenous cell sources from a therapeutic point of view. Because stem cells (DPSCs) are present in the dental pulp, the source of the cells depends on whether the vital pulp is preserved in the root canal. Pulpotomy is a dental procedure that removes the pulp of a tooth in the crown and leaves the pulp in the root canal as intact vital pulp. It has been mainly used for the normal development of the root by preserving pulp in immature teeth; however, it can also be performed on mature teeth. If the cell-homing strategy is used after pulpotomy, the DPSCs that exist in the immediate vicinity are mainly homing and can regenerate pulp dentin, an intrinsic ability under the influence of signaling molecules (Shi et al., 2020). However, it is known that in pulpectomy, which extirpates the entire pulp tissue, stem cells from apical papilla, and periodontal ligament stem cells, which are mainly located in the apical foramen, are homed (Liu J.-Y. et al., 2015; He et al., 2017).

The pulp tissue regeneration experiment using the cell-homing-based technique is mainly performed by transplanting human teeth into animal models. Briefly, after RCT of human extracted teeth, different types of signaling molecules are combined on various scaffolds and applied to empty root canals. The grafts are transplanted into animal models to evaluate pulp regeneration. Early studies mainly focused on signaling molecules. Pulp-like tissue has been reported to be regenerated in a mouse model using a combination of bFGF, PDGF (factors for cellular chemotaxis), NGF (for neural growth), VEGF (for angiogenesis), and BMP7 (for odontoblast

differentiation and mineralization) (Kim et al., 2010). Although the study by Kim et al. (2010) used an ectopic model, it is significant as a starting point for demonstrating a clinically accessible cell-homing approach for pulp regeneration in humans. Experiments were then conducted using a single molecule to determine which signaling molecule is most essential for pulp regeneration. Early research showed that pulp-like tissue was regenerated by injecting bFGF alone, suggesting that the signaling molecule that triggers stem cell recruitment plays the most important role (Suzuki et al., 2011). Another study found that there was no significant difference in the pulp regeneration effect between bFGF and G-CSF application and reported that bFGF could be replaced with G-CSF (Takeuchi et al., 2015). As a result of these studies, research was conducted mainly on signaling molecules that can home stem cells to the empty pulp space, and factors such as SDF-1 (Yang et al., 2015; Zhang L. X. et al., 2015) and stem cell factor (SCF) (Ruangsawasdi et al., 2017) have also been reported to be effective.

Cell-homing strategies have the advantage of not requiring isolation or manipulation of stem cells *in vitro*, making them more economical and may be easier to perform clinically. However, the cell-homing strategy also has some limitations. If the apical papilla or follicle is damaged due to severe inflammation accompanied by pulpal necrosis, it may be difficult to sustain the root development of immature teeth because there are not enough stem cells to support odontoblast differentiation or dentin formation. Likewise, even when cell-homing-based RET is performed on mature teeth, treatment may not be successful if there are not enough stem cells, and the outcome of treatment cannot be predicted because it is impossible to determine the status of stem cells in the apical papilla or dental follicle. Therefore, numerous aspects still need to be addressed to obtain applicable and predictable results in clinical practice. Despite the great advances in dental pulp regeneration through cell-homing-based RET over the past few years, further investigation and development are needed.

## Cell-Transplantation-Based Regenerative Endodontic Treatment

The basic concept of cell-transplantation-based RET is the transplantation of exogenous stem cells onto scaffolds with signaling molecules for tissue regeneration (Demarco et al., 2011) (**Figure 4D**). For RET, a cell transplantation strategy based on the classical concept of tissue engineering was first proposed for pulp tissue regeneration and has made remarkable progress. Based on the classical concept of tissue engineering, early experiments and continuous animal studies have been conducted to investigate the effect of stem cell implantation on pulp regeneration (Dissanayaka et al., 2015; Abbass et al., 2020; Ahmed et al., 2020). In a preliminary study in which autologous DPSCs were transplanted together with Gelfoam as a scaffold for immature permanent incisors of canines, it was found that pulp-like tissues, including dentin-like tissues and blood vessels, were regenerated (Wang et al., 2013). Another study reported that autologous DPSCs with platelet-rich fibrin promoted the regeneration of pulp-dentin like tissue in dogs (Chen et al., 2015). Further animal studies have shown that human DPSCs, along with platelet-derived growth factor, have successfully induced pulp-like tissue by applying them to the empty pulp space of rats (Cai et al., 2016). A study in which a chitosan hydrogel scaffold containing autologous DPSCs and growth factors was applied to immature necrotic permanent teeth with apical periodontitis in dogs confirmed that root maturation was complete histologically and radiologically, as well as regeneration of pulp and dentin-like tissues (El Ashiry et al., 2018).

In cell-transplantation-based RET, various scaffolds have been used to allow the applied stem cells to promote attachment, proliferation, and differentiation. Gelfoam was used as a scaffold for applying autologous DPSCs in dogs (Wang et al., 2013), and an injectable nanopeptide hydrogel was also used to apply porcine DPSCs (Mangione et al., 2017). In addition, it was reported that the gelatin-based scaffold was histologically and radiologically more effective than the fibrin-based scaffold when human DPSCs were placed on gelatin- or fibrin-based scaffolds and transplanted into minipigs (Jang et al., 2020). Furthermore, to use the tissue most similar to the original tissue as a scaffold, decellularized swine dental pulp tissue was used as a scaffold and applied to human DPSCs, and the regeneration of pulp-like tissue was confirmed histologically (Hu et al., 2017). In addition, scaffold-free RET, which can replace the role of scaffolds by constructing cells in three dimensions, is being studied. Pulp-like tissue regeneration was achieved by transforming canine DPSCs into cell sheet fragments and applying them along with the signaling molecule platelet-rich fibrin (Chen et al., 2015). In addition, it was confirmed that the pulp-like tissue was regenerated by subcutaneously implanting human DPSCs into three-dimensional (3D) cell constructs without signaling molecules or scaffolds in immunodeficient mice (Itoh et al., 2018). A recent study revealed that 3D cell sheets could enhance the therapeutic potency of MSCs, suggesting the possibility that cell sheets could replace scaffolds (Bou-Ghannam et al., 2021).

In cell-transplantation-based RET, autologous stem cells have been used in many animal studies, and all clinical trials performed using this method yielded successful results (Kim, 2021). Therefore, the cell-transplantation-based RET method appears to be the most appropriate for clinical applications. In addition, because stem cells with suitable characteristics are directly applied together to induce pulp-like tissue, the limitation of stem cell induction, as in cell-homing-based RET, can be overcome. However, a major obstacle to autologous stem cell transplantation is the availability of pulp tissue. The ethically best available methods for isolating DPSCs required for pulp regeneration are wisdom teeth or orthodontic extraction teeth. It is difficult to extract wisdom teeth or perform orthodontic treatment to treat irreversible pulpitis. In addition, the scaffolds and signaling molecules used in each study were slightly different; therefore, more research on the best combination is needed. Collectively, cell-transplantation-based RET in dentistry for dentin pulp tissue regeneration still faces challenges. Future strategies should be directed toward creating a

suitable regenerative microenvironment using an ideal combination of signaling molecules and scaffolds that are most suitable for pulp tissue regeneration (Mangione et al., 2017).

## ALLOGENEIC TRANSPLANTATION

Stem cell therapy has been proposed as an effective regenerative technology to restore the function of teeth that have lost function due to pulpitis. Several specific regeneration methods have been introduced, and autologous transplantation of a subpopulation of DPSCs (pulp-derived $CD31^-$ SP cells and pulp-derived $CD105^+$ cells treated with SDF-1) have been successful in demonstrating the possibility of pulp regeneration (Iohara et al., 2011; Ishizaka et al., 2012). Subsequently, transplantation of DPSCs with autologous platelet-rich fibrin successfully regenerated pulp-like tissue and induced the deposition of regenerated dentin (Chen et al., 2015). In a preclinical study, the safety and efficacy of autologous DPSC transplantation therapy were demonstrated by harvesting and culturing DPSCs under good manufacturing practice conditions and applying them along with G-CSF (Iohara et al., 2013). Moreover, a recent human clinical study suggested that autologous DPSC transplantation is safe and may effectively induce pulp regeneration (Nakashima et al., 2017). Although autologous DPSC transplantation has demonstrated some efficacy and potential for tissue regeneration, certain limitations still need to be overcome, the biggest limitation being the presence of unnecessary teeth, such as wisdom teeth, to regenerate the pulp of a specific tooth with autologous DPSC transplantation. This limitation is particularly noticeable in elderly patients since there is a high probability that elderly patients do not already possess unneeded teeth. Even if elderly patients have usable teeth for transplantation, their DPSCs may show dysfunction due to aging. A recent study reported that DPSCs exhibit typical senescence features, such as reduced proliferation, reduced differentiation potential, and enlarged cell shape with aging (Yi et al., 2017). In addition, osteogenic potential decreases in aged human DPSCs (Yi et al., 2017; Iezzi

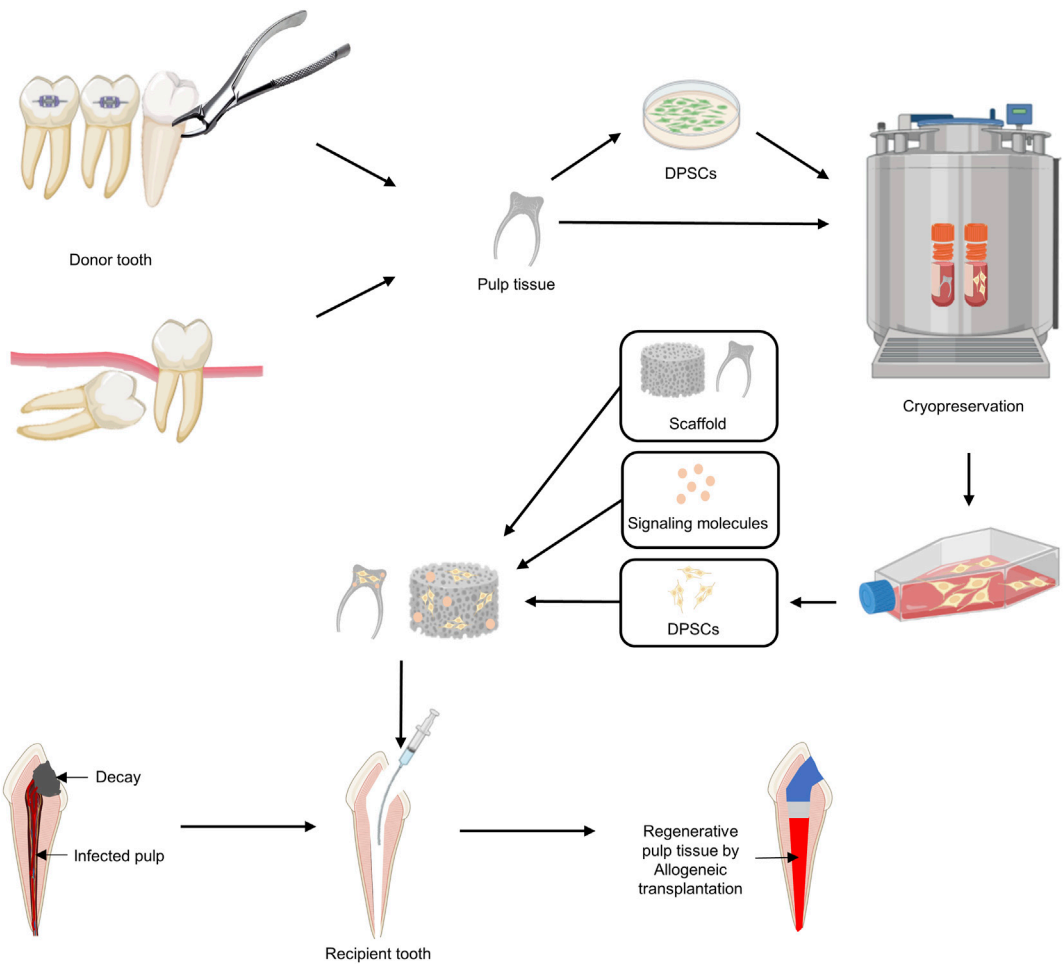


**FIGURE 5 |** A proposed strategy using allogeneic DPSC transplantation for pulp regeneration. 1) Dental pulp tissue is removed from an uninfected orthodontic extraction tooth or wisdom tooth (donor). 2) After isolating and culturing DPSCs from dental pulp, cryopreservation of DPSCs or dental pulp itself is performed. 3) DPSCs are expanded using cryopreserved DPSCs or dental pulp tissues according to the patient's treatment plan for pulpectomy. 4) Signaling molecules and DPSCs are added to the appropriate scaffold and applied to the disinfected pulp space.

et al., 2019), and the expression of dentin matrix acidic phosphoprotein 1 and dentin sialophosphoprotein, key markers of odontogenic differentiation, decreases with age (Iezzi et al., 2019). Various studies have proven that neurogenic potential, which is one of the essential factors for pulp regeneration, also decreases with age (Martens et al., 2012; Feng et al., 2013). However, it is not efficient for individual patients to bank DPSCs at an early age and store them until needed. Moreover, the storage, safety, and quality control costs are high. Therefore, allogeneic DPSC transplantation, which is stored whenever unwanted teeth are found in young patients and applied to patients in need, saves time and cost, and is beneficial for quality control (Collart-Dutilleul et al., 2015). Therefore, cell banks are essential to overcome the limitations of autologous transplantation and to successfully perform allogeneic transplantation. Many studies have shown that DPSCs can be cryopreserved without significant cell damage (Zhang et al., 2006; Gioventu et al., 2012). In addition, tissue cryopreservation, which is economically advantageous because it reduces the time and money required to isolate and incubate cells, has been shown to have no effect on cells (Han et al., 2017). Moreover, tissue cryopreservation for more than 1 year is likely to significantly reduce the cost of autologous cell-transplantation-based RET. Notably, systematizing the collection, banking, and application of pulp tissue are expected to reduce costs and simplify clinical application procedures.

A concern with the use of allogeneic cells is that immune rejection may occur in the host due to a MHC mismatch. The reason that allogeneic MSCs can be applied despite these concerns is that MSCs themselves have low immunogenicity and immunosuppressive properties. The low immunogenicity of MSCs is attributed to the low expression of class I MHC (MHC-I) proteins and costimulatory molecules and lack of expression of MHC-II proteins (Pittenger et al., 1999; Le Blanc et al., 2003). Therefore, MSCs do not exhibit cytotoxic effects on host immune cells (Jones and McTaggart, 2008) and have the advantage of being able to perform transplantation without considering MHC (Ankrum et al., 2014). MSCs and DPSCs exhibit low immunogenicity and can induce immune tolerance in the host (Iohara et al., 2018). Moreover, although the mechanism is still controversial, the immunomodulatory properties of DPSCs increase their potential as a source for allogeneic transplantation. This immunosuppressive property suggests that even if the host's immune response occurs during allogeneic transplantation by other factors, it can be overcome, and transplantation can be successful. Based on these characteristics of DPSCs, it was recently reported that the transplantation of allogeneic DPSCs in dogs was successfully performed (Iohara et al., 2018). In this study, allogeneic transplantation DPSCs mismatched with dog leukocyte antigen (DLA) did not show toxicity and showed similar effects to DLA-matched DPSCs in pulp tissue regeneration. Thus, allogeneic "off-the-self" therapies can achieve the goal of clinical stem cell-based therapy to maintain long-term stability by inducing universal cell donor adoption, banking donated cells, and timely delivery of appropriate cells for patients in need (Telukuntla et al., 2013; Heathman et al., 2015).

## CONCLUSION

One of the challenges facing modern dentistry is not only to remove the infected pulp but also to maintain the pulp so that it can regain vitality (Miran et al., 2016; Yang et al., 2016). The goal of pulp regenerative treatment for infected pulp is to restore it functionally by reconstituting the pulp–dentin complex (Mao et al., 2012; He et al., 2019), but it seems difficult to achieve this goal with current clinical protocols (Kim et al., 2018). With the development of regenerative medicine using stem cells, cell-transplantation-based regeneration protocols for pulp regeneration have been steadily developed. The discovery and characterization of dental MSCs, especially DPSCs, raise expectations for pulp regeneration in RET in the future. Transplantation of DPSCs, which induce neuroangiogenesis, has achieved complete pulp regeneration in several studies (Murakami et al., 2015; Nakashima et al., 2017), with the crucial achievement of regeneration of neuroangiogenesis to achieve functional restoration of dental pulp. Nevertheless, it should be noted that current pulp regeneration is based only on autologous DPSCs. Transplantation with allogeneic DPSCs or pulp tissue from cryo-preserved cells or tissue banking clouds is an ideal clinical approach for pulp regeneration against infected pulp, especially in aged patients (**Figure 5**). Optimizing disinfection procedures as well as the application of proper scaffolds and/or factors for promoting neuroangiogenesis should also be key factors for allogeneic transplantation-induced pulp regeneration. Given the scientific evidence to date, cell-transplantation-based RET for pulp regeneration has been accepted as a promising treatment protocol. In addition, a functional pulp regeneration strategy through neurovascularization has the potential to become an innovative model for regenerative medicine and not only for dental pulp regeneration.

## AUTHOR CONTRIBUTIONS

KK and H-WL contributed towards the planning of this review. All authors contributed to the article and gave their final approval and agree to be accountable for all aspects of the work.

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## REFERENCES


Abbass, M. M. S., El-Rashidy, A. A., Sadek, K. M., Moshy, S. E., Radwan, I. A., Rady, D., et al. (2020). Hydrogels and Dentin-Pulp Complex Regeneration: From the Benchtop to Clinical Translation. *Polymers* 12 (12), 2935. doi:10.3390/polym12122935

Ahmed, G. M., Abouauf, E. A., AbuBakr, N., Dörfer, C. E., and El-Sayed, K. F. (2020). Tissue Engineering Approaches for Enamel, Dentin, and Pulp Regeneration: An Update. *Stem Cell Int.* 2020, 1–15. doi:10.1155/2020/5734539

Ankrum, J. A., Ong, J. F., and Karp, J. M. (2014). Mesenchymal Stem Cells: Immune Evasive, Not Immune Privileged. *Nat. Biotechnol.* 32 (3), 252–260. doi:10.1038/nbt.2816

Apel, C., Forlenza, O. V., de Paula, V. J. R., Talib, L. L., Denecke, B., Eduardo, C. P., et al. (2009). The Neuroprotective Effect of Dental Pulp Cells in Models of Alzheimer's and Parkinson's Disease. *J. Neural Transm.* 116 (1), 71–78. doi:10.1007/s00702-008-0135-3

Arthur, A., Rychkov, G., Shi, S., Koblar, S. A., and Gronthos, S. (2008). Adult Human Dental Pulp Stem Cells Differentiate toward Functionally Active Neurons Under Appropriate Environmental Cues. *Stem Cells* 26 (7), 1787–1795. doi:10.1634/stemcells.2007-0979

Bai, Y., Bai, Y., Matsuzaka, K., Hashimoto, S., Fukuyama, T., Wu, L., et al. (2011). Cementum- and Periodontal Ligament-Like Tissue Formation by Dental Follicle Cell Sheets Co-Cultured with Hertwig's Epithelial Root Sheath Cells. *Bone* 48 (6), 1417–1426. doi:10.1016/j.bone.2011.02.016

Bakhtiar, H., Rajabi, S., Pezeshki-Modaress, M., Ellini, M. R., Panahinia, M., Alijani, S., et al. (2021). Optimizing Methods for Bovine Dental Pulp Decellularization. *J. Endod.* 47 (1), 62–68. doi:10.1016/j.joen.2020.08.027

Banchs, F., and Trope, M. (2004). Revascularization of Immature Permanent Teeth with Apical Periodontitis: New Treatment Protocol? *J. Endod.* 30 (4), 196–200. doi:10.1097/00004770-200404000-00003

Bastos, A. d. C., de Oliveira, J. B., Mello, K. F. R., Leão, P. B., Artese, F., and Normando, D. (2016). The Ability of Orthodontists and Oral/Maxillofacial Surgeons to Predict Eruption of Lower Third Molar. *Prog. Orthod.* 17 (1), 21. doi:10.1186/s40510-016-0134-0

Becerra, P., Ricucci, D., Loghin, S., Gibbs, J. L., and Lin, L. M. (2014). Histologic Study of a Human Immature Permanent Premolar with Chronic Apical Abscess after Revascularization/Revitalization. *J. Endod.* 40 (1), 133–139. doi:10.1016/j.joen.2013.07.017

Bergenholtz, G., Cox, C. F., Loesche, W. J., and Syed, S. A. (1982). Bacterial Leakage Around Dental Restorations: its Effect on the Dental Pulp. *J. Oral Pathol. Med.* 11 (6), 439–450. doi:10.1111/j.1600-0714.1982.tb00188.x

Bianco, P., Cao, X., Frenette, P. S., Mao, J. J., Robey, P. G., Simmons, P. J., et al. (2013). The Meaning, the Sense and the Significance: Translating the Science of Mesenchymal Stem Cells Into Medicine. *Nat. Med.* 19 (1), 35–42. doi:10.1038/nm.3028

Bianco, P., Riminucci, M., Gronthos, S., and Robey, P. G. (2001). Bone Marrow Stromal Stem Cells: Nature, Biology, and Potential Applications. *Stem Cells* 19 (3), 180–192. doi:10.1634/stemcells.19-3-180

Bou-Ghannam, S., Kim, K., Grainger, D. W., and Okano, T. (2021). 3D Cell Sheet Structure Augments Mesenchymal Stem Cell Cytokine Production. *Sci. Rep.* 11 (1), 8170. doi:10.1038/s41598-021-87571-7

Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L. (1994). Treatment of Deep Cartilage Defects in the Knee with Autologous Chondrocyte Transplantation. *N. Engl. J. Med.* 331 (14), 889–895. doi:10.1056/NEJM199410063311401

Bronckaers, A., Hilkens, P., Fanton, Y., Struys, T., Gervois, P., Politis, C., et al. (2013). Angiogenic Properties of Human Dental Pulp Stem Cells. *PLoS One* 8 (8), e71104. doi:10.1371/journal.pone.0071104

Cai, S., Zhang, W., and Chen, W. (2016). PDGFRβ+/c-kit+ Pulp Cells Are Odontoblastic Progenitors Capable of Producing Dentin-like Structure *In Vitro* and *In Vivo*. *BMC Oral Health* 16 (1), 113. doi:10.1186/s12903-016-0307-8

Chang, C.-C., Chang, K.-C., Tsai, S.-J., Chang, H.-H., and Lin, C.-P. (2014). Neurogenic Differentiation of Dental Pulp Stem Cells to Neuron-like Cells in Dopaminergic and Motor Neuronal Inductive media. *J. Formos. Med. Assoc.* 113 (12), 956–965. doi:10.1016/j.jfma.2014.09.003

Chang, Y.-C., Huang, F.-M., Tai, K.-W., and Chou, M.-Y. (2001). The Effect of Sodium Hypochlorite and Chlorhexidine on Cultured Human Periodontal Ligament Cells. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 92 (4), 446–450. doi:10.1067/moe.2001.116812

Chen, F.-M., Sun, H.-H., Lu, H., and Yu, Q. (2012). Stem Cell-Delivery Therapeutics for Periodontal Tissue Regeneration. *Biomaterials* 33 (27), 6320–6344. doi:10.1016/j.biomaterials.2012.05.048

Chen, Y.-J., Zhao, Y.-H., Zhao, Y.-J., Liu, N.-X., Lv, X., Li, Q., et al. (2015). Potential Dental Pulp Revascularization and Odonto-/Osteogenic Capacity of a Novel Transplant Combined with Dental Pulp Stem Cells and Platelet-Rich Fibrin. *Cell Tissue Res* 361 (2), 439–455. doi:10.1007/s00441-015-2125-8

Collart-Dutilleul, P. Y., Chaubron, F., De Vos, J., and Cuisinier, F. J. (2015). Allogenic Banking of Dental Pulp Stem Cells for Innovative Therapeutics. *World J. Stem Cell* 7 (7), 1010–1021. doi:10.4252/wjsc.v7.i7.1010

Cooper, P. R., Takahashi, Y., Graham, L. W., Simon, S., Imazato, S., and Smith, A. J. (2010). Inflammation-Regeneration Interplay in the Dentine-Pulp Complex. *J. Dent.* 38 (9), 687–697. doi:10.1016/j.jdent.2010.05.016

Cordeiro, M. M., Dong, Z., Kaneko, T., Zhang, Z., Miyazawa, M., Shi, S., et al. (2008). Dental Pulp Tissue Engineering with Stem Cells from Exfoliated Deciduous Teeth. *J. Endod.* 34 (8), 962–969. doi:10.1016/j.joen.2008.04.009

Cvek, M. (1992). Prognosis of Luxated Non-vital Maxillary Incisors Treated with Calcium Hydroxide and Filled with Gutta-Percha. A Retrospective Clinical Study. *Dent Traumatol.* 8 (2), 45–55. doi:10.1111/j.1600-9657.1992.tb00228.x

Daley, G. Q. (2012). The Promise and Perils of Stem Cell Therapeutics. *Cell Stem Cell* 10 (6), 740–749. doi:10.1016/j.stem.2012.05.010

Davidson, R. M. (1994). Neural Form of Voltage-Dependent Sodium Current in Human Cultured Dental Pulp Cells. *Arch. Oral Biol.* 39 (7), 613–620. doi:10.1016/0003-9969(94)90137-6

de Chevigny, C., Dao, T. T., Basrani, B. R., Marquis, V., Farzaneh, M., Abitbol, S., et al. (2008). Treatment Outcome in Endodontics: The Toronto Study-phase 4: Initial Treatment. *J. Endod.* 34 (3), 258–263. doi:10.1016/j.joen.2007.10.017

Demarco, F. F., Conde, M. C. M., Cavalcanti, B. N., Casagrande, L., Sakai, V. T., and Nör, J. E. (2011). Dental Pulp Tissue Engineering. *Braz. Dent. J.* 22 (1), 3–13. doi:10.1590/s0103-64402011000100001

Demircan, P. C., Sariboyaci, A. E., Unal, Z. S., Gacar, G., Subasi, C., and Karaoz, E. (2011). Immunoregulatory Effects of Human Dental Pulp-Derived Stem Cells on T Cells: Comparison of Transwell Co-Culture and Mixed Lymphocyte Reaction Systems. *Cytotherapy* 13 (10), 1205–1220. doi:10.3109/14653249.2011.605351

Dimitrova-Nakov, S., Baudry, A., Harichane, Y., Kellermann, O., Goldberg, M., and Dr es Sciences, N. (2014). Pulp Stem Cells: Implication in Reparative Dentin Formation. *J. Endod.* 40 (4 Suppl. l), S13–S18. doi:10.1016/j.joen.2014.01.011

Ding, G., Liu, Y., Wang, W., Wei, F., Liu, D., Fan, Z., et al. (2010). Allogeneic Periodontal Ligament Stem Cell Therapy for Periodontitis in Swine. *Stem Cells* 28 (10), 1829–1838. doi:10.1002/stem.512

Dissanayaka, W. L., Hargreaves, K. M., Jin, L., Samaranayake, L. P., and Zhang, C. (2015). The Interplay of Dental Pulp Stem Cells and Endothelial Cells in an Injectable Peptide Hydrogel on Angiogenesis and Pulp Regeneration *In Vivo*. *Tissue Eng. A* 21 (3-4), 550–563. doi:10.1089/ten.TEA.2014.0154

Dissanayaka, W. L., Zhan, X., Zhang, C., Hargreaves, K. M., Jin, L., and Tong, E. H. Y. (2012). Coculture of Dental Pulp Stem Cells with Endothelial Cells Enhances Osteo-/odontogenic and Angiogenic Potential *In Vitro*. *J. Endod.* 38 (4), 454–463. doi:10.1016/j.joen.2011.12.024

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S., et al. (2006). Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy Position Statement. *Cytotherapy* 8 (4), 315–317. doi:10.1080/14653240600855905

Du, C., and Moradian-Oldak, J. (2006). Tooth Regeneration: Challenges and Opportunities for Biomedical Material Research. *Biomed. Mater.* 1 (1), R10–R17. doi:10.1088/1748-6041/1/1/R02

El Ashiry, E. A., Alamoudi, N. M., El Ashiry, M. K., Bastawy, H. A., El Derwi, D. A., and Atta, H. M. (2018). Tissue Engineering of Necrotic Dental Pulp of Immature Teeth with Apical Periodontitis in Dogs: Radiographic and Histological Evaluation. *J. Clin. Pediatr. Dent* 42 (5), 373–382. doi:10.17796/1053-4625-42.5.9

European Society of Endodontology (2006). Quality Guidelines for Endodontic Treatment: Consensus Report of the European Society of Endodontology. *Int. Endod. J.* 39 (12), 921–930. doi:10.1111/j.1365-2591.2006.01180.x

Farges, J.-C., Alliot-Licht, B., Renard, E., Ducret, M., Gaudin, A., Smith, A. J., et al. (2015). Dental Pulp Defence and Repair Mechanisms in Dental Caries. *Mediators Inflamm.* 2015, 1–16. doi:10.1155/2015/230251

Fawzy El-Sayed, K. M., Jakusz, K., Jochens, A., Dörfer, C., and Schwendicke, F. (2015). Stem Cell Transplantation for Pulpal Regeneration: A Systematic Review. *Tissue Eng. B: Rev.* 21 (5), 451–460. doi:10.1089/ten.TEB.2014.0675

Feng, X., Xing, J., Feng, G., Sang, A., Shen, B., Xu, Y., et al. (2013). Age-Dependent Impaired Neurogenic Differentiation Capacity of Dental Stem Cell is Associated with Wnt/β-Catenin Signaling. *Cell Mol Neurobiol* 33 (8), 1023–1031. doi:10.1007/s10571-013-9965-0

Fernandes, G., and Yang, S. (2016). Application of Platelet-Rich Plasma with Stem Cells in Bone and Periodontal Tissue Engineering. *Bone Res.* 4, 16036. doi:10.1038/boneres.2016.36

Fleming, E., and Afful, J. (2018). Prevalence of Total and Untreated Dental Caries Among Youth: United States, 2015-2016. *NCHS Data Brief* 2018 (307), 1–8.

Frenette, P. S., Pinho, S., Lucas, D., and Scheiermann, C. (2013). Mesenchymal Stem Cell: keystone of the Hematopoietic Stem Cell Niche and a Stepping-Stone for Regenerative Medicine. *Annu. Rev. Immunol.* 31, 285–316. doi:10.1146/annurev-immunol-032712-095919

Frozoni, M., Zaia, A. A., Line, S. R. P., and Mina, M. (2012). Analysis of the Contribution of Nonresident Progenitor Cells and Hematopoietic Cells to Reparative Dentinogenesis Using Parabiosis Model in Mice. *J. Endod.* 38 (9), 1214–1219. doi:10.1016/j.joen.2012.05.016

Galler, K. M. (2016). Clinical Procedures for Revitalization: Current Knowledge and Considerations. *Int. Endod. J.* 49 (10), 926–936. doi:10.1111/iej.12606

Galler, K. M., D'Souza, R. N., Federlin, M., Cavender, A. C., Hartgerink, J. D., Hecker, S., et al. (2011). Dentin Conditioning Codetermines Cell Fate in Regenerative Endodontics. *J. Endod.* 37 (11), 1536–1541. doi:10.1016/j.joen.2011.08.027

Gandia, C., Armiñan, A., García-Verdugo, J. M., Lledó, E., Ruiz, A., Miñana, M. D., et al. (2008). Human Dental Pulp Stem Cells Improve Left Ventricular Function, Induce Angiogenesis, and Reduce Infarct Size in Rats with Acute Myocardial Infarction. *Stem Cells* 26 (3), 638–645. doi:10.1634/stemcells.2007-0484

Gao, C., Peng, S., Feng, P., and Shuai, C. (2017). Bone Biomaterials and Interactions with Stem Cells. *Bone Res.* 5, 17059. doi:10.1038/boneres.2017.59

Gioventù, S., Andriolo, G., Bonino, F., Frasca, S., Lazzari, L., Montelatici, E., et al. (2012). A Novel Method for Banking Dental Pulp Stem Cells. *Transfus. Apher. Sci.* 47 (2), 199–206. doi:10.1016/j.transci.2012.06.005

Gnanasegaran, N., Govindasamy, V., Simon, C., Gan, Q. F., Vincent-Chong, V. K., Mani, V., et al. (2017). Effect of Dental Pulp Stem Cells in MPTP-Induced Old-Aged Mice Model. *Eur. J. Clin. Invest.* 47 (6), 403–414. doi:10.1111/eci.12753

Goldberg, M., and Smith, A. J. (2004). Cells and Extracellular Matrices of Dentin and Pulp: A Biological Basis for Repair and Tissue Engineering. *Crit. Rev. Oral Biol. Med.* 15 (1), 13–27. doi:10.1177/154411130401500103

Grayson, W. L., Bunnell, B. A., Martin, E., Frazier, T., Hung, B. P., and Gimble, J. M. (2015). Stromal Cells and Stem Cells in Clinical Bone Regeneration. *Nat. Rev. Endocrinol.* 11 (3), 140–150. doi:10.1038/nrendo.2014.234

Gronthos, S., Brahim, J., Li, W., Fisher, L. W., Cherman, N., Boyde, A., et al. (2002). Stem Cell Properties of Human Dental Pulp Stem Cells. *J. Dent Res.* 81 (8), 531–535. doi:10.1177/154405910208100806

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., and Shi, S. (2000). Postnatal Human Dental Pulp Stem Cells (DPSCs) *In Vitro* and *In Vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 97 (25), 13625–13630. doi:10.1073/pnas.240309797

Guo, W., Chen, L., Gong, K., Ding, B., Duan, Y., and Jin, Y. (2012). Heterogeneous Dental Follicle Cells and the Regeneration of Complex Periodontal Tissues. *Tissue Eng. Part A* 18 (5–6), 459–470. doi:10.1089/ten.TEA.2011.0261

Guo, Y., Yuan, Y., Wu, L., Ho, T.-V., Jing, J., Sugii, H., et al. (2018). BMP-IHH-mediated Interplay between Mesenchymal Stem Cells and Osteoclasts Supports Calvarial Bone Homeostasis and Repair. *Bone Res.* 6, 30. doi:10.1038/s41413-018-0031-x

Halvorsen, Y., Wilkison, W., and Gimble, J. (2000). Adipose-derived Stromal Cells-Their Utility and Potential in Bone Formation. *Int. J. Obes.* 24 (Suppl. 4), S41–S44. doi:10.1038/sj.ijo.0801503

Han, C., Yang, Z., Zhou, W., Jin, F., Song, Y., Wang, Y., et al. (2010). Periapical Follicle Stem Cell: a Promising Candidate for Cementum/periodontal Ligament Regeneration and Bio-Root Engineering. *Stem Cell Dev.* 19 (9), 1405–1415. doi:10.1089/scd.2009.0277

Han, Y.-J., Kang, Y.-H., Shivakumar, S. B., Bharti, D., Son, Y.-B., Choi, Y.-H., et al. (2017). Stem Cells from Cryopreserved Human Dental Pulp Tissues Sequentially Differentiate into Definitive Endoderm and Hepatocyte-like Cells *In Vitro*. *Int. J. Med. Sci.* 14 (13), 1418–1429. doi:10.7150/ijms.22152

Handa, K., Saito, M., Tsunoda, A., Yamauchi, M., Hattori, S., Sato, S., et al. (2002). Progenitor Cells from Dental Follicle are Able to Form Cementum Matrix *In Vivo*. *Connect. Tissue Res.* 43 (2-3), 406–408. doi:10.1080/03008200290001023

Haynesworth, S. E., Goshima, J., Goldberg, V. M., and Caplan, A. I. (1992). Characterization of Cells with Osteogenic Potential from Human Marrow. *Bone* 13 (1), 81–88. doi:10.1016/8756-3282(92)90364-3

He, L., Kim, S. G., Gong, Q., Zhong, J., Wang, S., Zhou, X., et al. (2017). Regenerative Endodontics for Adult Patients. *J. Endod.* 43 (9S), S57–S64. doi:10.1016/j.joen.2017.06.012

He, L., Zhou, J., Chen, M., Lin, C.-S., Kim, S. G., Zhou, Y., et al. (2019). Parenchymal and Stromal Tissue Regeneration of Tooth Organ by Pivotal Signals Reinstated in Decellularized Matrix. *Nat. Mater.* 18 (6), 627–637. doi:10.1038/s41563-019-0368-6

Heathman, T. R., Nienow, A. W., McCall, M. J., Coopman, K., Kara, B., and Hewitt, C. J. (2015). The Translation of Cell-Based Therapies: Clinical Landscape and Manufacturing Challenges. *Regenerative Med.* 10 (1), 49–64. doi:10.2217/rme.14.73

Hong, J. W., Lim, J. H., Chung, C. J., Kang, T. J., Kim, T. Y., Kim, Y. S., et al. (2017). Immune Tolerance of Human Dental Pulp-Derived Mesenchymal Stem Cells Mediated by CD4+CD25+ FoxP3+ Regulatory T-Cells and Induced by TGF-B1 and IL-10. *Yonsei Med. J.* 58 (5), 1031–1039. doi:10.3349/ymj.2017.58.5.1031

Hossein-Khannazer, N., Hashemi, S. M., Namaki, S., Ghanbarian, H., Sattari, M., and Khojasteh, A. (2019). Study of the Immunomodulatory Effects of Osteogenic Differentiated Human Dental Pulp Stem Cells. *Life Sci.* 216, 111–118. doi:10.1016/j.lfs.2018.11.040

Hu, L., Gao, Z., Xu, J., Zhu, Z., Fan, Z., Zhang, C., et al. (2017). Decellularized Swine Dental Pulp as a Bioscaffold for Pulp Regeneration. *Biomed. Res. Int.* 2017, 1–9. doi:10.1155/2017/9342714

Huang, G. T.-J., Sonoyama, W., Liu, Y., Liu, H., Wang, S., and Shi, S. (2008). The Hidden Treasure in Apical Papilla: The Potential Role in Pulp/Dentin Regeneration and Bioroot Engineering. *J. Endod.* 34 (6), 645–651. doi:10.1016/j.joen.2008.03.001

Hunter, D. J., Bardet, C., Mouraret, S., Liu, B., Singh, G., Sadoine, J., et al. (2015). Wnt Acts as a Prosurvival Signal to Enhance Dentin Regeneration. *J. Bone Miner Res.* 30 (7), 1150–1159. doi:10.1002/jbmr.2444

Iezzi, I., Cerqueni, G., Licini, C., Lucarini, G., and Mattioli Belmonte, M. (2019). Dental Pulp Stem Cells Senescence and Regenerative Potential Relationship. *J. Cell Physiol.* 234 (5), 7186–7197. doi:10.1002/jcp.27472

Imura, N., Pinheiro, E. T., Gomes, B. P. F. A., Zaia, A. A., Ferraz, C. C. R., and Souza-Filho, F. J. (2007). The Outcome of Endodontic Treatment: A Retrospective Study of 2000 Cases Performed by a Specialist. *J. Endod.* 33 (11), 1278–1282. doi:10.1016/j.joen.2007.07.018

Iohara, K., Imabayashi, K., Ishizaka, R., Watanabe, A., Nabekura, J., Ito, M., et al. (2011). Complete Pulp Regeneration After Pulpectomy by Transplantation of CD105+ Stem Cells with Stromal Cell-Derived Factor-1. *Tissue Eng. Part A* 17 (15–16), 1911–1920. doi:10.1089/ten.TEA.2010.0615

Iohara, K., Murakami, M., Takeuchi, N., Osako, Y., Ito, M., Ishizaka, R., et al. (2013). A Novel Combinatorial Therapy with Pulp Stem Cells and Granulocyte Colony-Stimulating Factor for Total Pulp Regeneration. *Stem Cell Transl. Med.* 2 (7), 521–533. doi:10.5966/sctm.2012-0132

Iohara, K., Utsunomiya, S., Kohara, S., and Nakashima, M. (2018). Allogeneic Transplantation of Mobilized Dental Pulp Stem Cells with the Mismatched Dog Leukocyte Antigen Type Is Safe and Efficacious for Total Pulp Regeneration. *Stem Cell Res. Ther.* 9 (1), 116. doi:10.1186/s13287-018-0855-8

Iohara, K., Zheng, L., Wake, H., Ito, M., Nabekura, J., Wakita, H., et al. (2008). A Novel Stem Cell Source for Vasculogenesis in Ischemia: Subfraction of Side Population Cells from Dental Pulp. *Stem Cells* 26 (9), 2408–2418. doi:10.1634/stemcells.2008-0393

Ishizaka, R., Iohara, K., Murakami, M., Fukuta, O., and Nakashima, M. (2012). Regeneration of Dental Pulp Following Pulpectomy by Fractionated Stem/progenitor Cells from Bone Marrow and Adipose Tissue. *Biomaterials* 33 (7), 2109–2118. doi:10.1016/j.biomaterials.2011.11.056

Itoh, Y., Sasaki, J. I., Hashimoto, M., Katata, C., Hayashi, M., and Imazato, S. (2018). Pulp Regeneration by 3-dimensional Dental Pulp Stem Cell Constructs. *J. Dent Res.* 97 (10), 1137–1143. doi:10.1177/0022034518772260

Iwaya, S.-i., Ikawa, M., and Kubota, M. (2001). Revascularization of an Immature Permanent Tooth with Apical Periodontitis and Sinus Tract. *Dent Traumatol.* 17 (4), 185–187. doi:10.1034/j.1600-9657.2001.017004185.x

Jackson, T. H., Guez, C., Lin, F.-C., Proffit, W. R., and Ko, C.-C. (2017). Extraction Frequencies at a university Orthodontic Clinic in the 21st Century: Demographic and Diagnostic Factors Affecting the Likelihood of Extraction. *Am. J. Orthod. Dent. Orthop.* 151 (3), 456–462. doi:10.1016/j.ajodo.2016.08.021

Jain, R. K., Au, P., Tam, J., Duda, D. G., and Fukumura, D. (2005). Engineering Vascularized Tissue. *Nat. Biotechnol.* 23 (7), 821–823. doi:10.1038/nbt0705-821

Janebodin, K., Zeng, Y., Buranaphatthana, W., Ieronimakis, N., and Reyes, M. (2013). VEGFR2-Dependent Angiogenic Capacity of Pericyte-Like Dental Pulp Stem Cells. *J. Dent Res.* 92 (6), 524–531. doi:10.1177/0022034513485599

Jang, J.-H., Moon, J.-H., Kim, S. G., and Kim, S.-Y. (2020). Pulp Regeneration with Hemostatic Matrices as a Scaffold in an Immature Tooth Minipig Model. *Sci. Rep.* 10 (1), 12536. doi:10.1038/s41598-020-69437-6

Jeanneau, C., Lundy, F. T., El Karim, I. A., and About, I. (2017). Potential Therapeutic Strategy of Targeting Pulp Fibroblasts in Dentin-Pulp Regeneration. *J. Endod.* 43 (9S), S17–S24. doi:10.1016/j.joen.2017.06.007

Jones, B. J., and McTaggart, S. J. (2008). Immunosuppression by Mesenchymal Stromal Cells: from Culture to Clinic. *Exp. Hematol.* 36 (6), 733–741. doi:10.1016/j.exphem.2008.03.006

Kassebaum, N. J., Bernabé, E., Dahiya, M., Bhandari, B., Murray, C. J. L., and Marcenes, W. (2015). Global Burden of Untreated Caries. *J. Dent Res.* 94 (5), 650–658. doi:10.1177/0022034515573272

Kémoun, P., Laurencin-Dalicieux, S., Rue, J., Farges, J.-C., Gennero, I., Conte-Auriol, F., et al. (2007). Human Dental Follicle Cells Acquire Cementoblast Features under Stimulation by BMP-2/-7 and Enamel Matrix Derivatives (EMD) *In Vitro*. *Cell Tissue Res.* 329 (2), 283–294. doi:10.1007/s00441-007-0397-3

Kim, J. Y., Xin, X., Moioli, E. K., Chung, J., Lee, C. H., Chen, M., et al. (2010). Regeneration of Dental-pulp-like Tissue by Chemotaxis-Induced Cell Homing. *Tissue Eng. Part A* 16 (10), 3023–3031. doi:10.1089/ten.TEA.2010.0181

Kim, S. G. (2021). A Cell-Based Approach to Dental Pulp Regeneration Using Mesenchymal Stem Cells: A Scoping Review. *Int. J. Med. Sci.* 22 (9), 4357. doi:10.3390/ijms22094357

Kim, S. G., Malek, M., Sigurdsson, A., Lin, L. M., and Kahler, B. (2018). Regenerative Endodontics: A Comprehensive Review. *Int. Endod. J.* 51 (12), 1367–1388. doi:10.1111/iej.12954

Kunimatsu, R., Nakajima, K., Awada, T., Tsuka, Y., Abe, T., Ando, K., et al. (2018). Comparative Characterization of Stem Cells from Human Exfoliated Deciduous Teeth, Dental Pulp, and Bone Marrow-Derived Mesenchymal Stem Cells. *Biochem. Biophys. Res. Commun.* 501 (1), 193–198. doi:10.1016/j.bbrc.2018.04.213

Kwack, K. H., Lee, J. M., Park, S. H., and Lee, H. W. (2017). Human Dental Pulp Stem Cells Suppress Alloantigen-Induced Immunity by Stimulating T Cells to Release Transforming Growth Factor Beta. *J. Endod.* 43 (1), 100–108. doi:10.1016/j.joen.2016.09.005

Laino, G., d'Aquino, R., Graziano, A., Lanza, V., Carinci, F., Naro, F., et al. (2005). A New Population of Human Adult Dental Pulp Stem Cells: a Useful Source of Living Autologous Fibrous Bone Tissue (LAB). *J. Bone Miner Res.* 20 (8), 1394–1402. doi:10.1359/JBMR.050325

Le Blanc, K., Tammik, C., Rosendahl, K., Zetterberg, E., and Ringdén, O. (2003). HLA Expression and Immunologic Propertiesof Differentiated and Undifferentiated Mesenchymal Stem Cells. *Exp. Hematol.* 31 (10), 890–896. doi:10.1016/s0301-472x(03)00110-3

Liu, J.-Y., Chen, X., Yue, L., Huang, G. T.-J., and Zou, X.-Y. (2015). CXC Chemokine Receptor 4 is Expressed Paravascularly in Apical Papilla and Coordinates with Stromal Cell-Derived Factor-1α during Transmigration of Stem Cells from Apical Papilla. *J. Endod.* 41 (9), 1430–1436. doi:10.1016/j.joen.2015.04.006

Liu, S., Liu, D., Chen, C., Hamamura, K., Moshaverinia, A., Yang, R., et al. (2015). MSC Transplantation Improves Osteopenia via Epigenetic Regulation of Notch Signaling in Lupus. *Cell Metab.* 22 (4), 606–618. doi:10.1016/j.cmet.2015.08.018

Liu, Y., Yang, R., Liu, X., Zhou, Y., Qu, C., Kikuiri, T., et al. (2014). Hydrogen Sulfide Maintains Mesenchymal Stem Cell Function and Bone Homeostasis via Regulation of Ca2+ Channel Sulfhydration. *Cell Stem Cell* 15 (1), 66–78. doi:10.1016/j.stem.2014.03.005

Liu, Y., Zheng, Y., Ding, G., Fang, D., Zhang, C., Bartold, P. M., et al. (2008). Periodontal Ligament Stem Cell-Mediated Treatment for Periodontitis in Miniature Swine. *Stem Cells* 26 (4), 1065–1073. doi:10.1634/stemcells.2007-0734

Lovelace, T. W., Henry, M. A., Hargreaves, K. M., and Diogenes, A. (2011). Evaluation of the Delivery of Mesenchymal Stem Cells into the Root Canal Space of Necrotic Immature Teeth after Clinical Regenerative Endodontic Procedure. *J. Endod.* 37 (2), 133–138. doi:10.1016/j.joen.2010.10.009

Luo, L., He, Y., Wang, X., Key, B., Lee, B. H., Li, H., et al. (2018). Potential Roles of Dental Pulp Stem Cells in Neural Regeneration and Repair. *Stem Cell Int.* 2018, 1–15. doi:10.1155/2018/1731289

Ma, L., Makino, Y., Yamaza, H., Akiyama, K., Hoshino, Y., Song, G., et al. (2012). Cryopreserved Dental Pulp Tissues of Exfoliated Deciduous Teeth Is a Feasible Stem Cell Resource for Regenerative Medicine. *PLoS One* 7 (12), e51777. doi:10.1371/journal.pone.0051777

Mangione, F., EzEldeen, M., Bardet, C., Lesieur, J., Bonneau, M., Decup, F., et al. (2017). Implanted Dental Pulp Cells Fail to Induce Regeneration in Partial Pulpotomies. *J. Dent Res.* 96 (12), 1406–1413. doi:10.1177/0022034517725523

Mao, J. J., Kim, S. G., Zhou, J., Ye, L., Cho, S., Suzuki, T., et al. (2012). Regenerative Endodontics. *Dent. Clin. North Am.* 56 (3), 639–649. doi:10.1016/j.cden.2012.05.005

Martens, W., Bronckaers, A., Politis, C., Jacobs, R., and Lambrichts, I. (2013). Dental Stem Cells and Their Promising Role in Neural Regeneration: an Update. *Clin. Oral Invest.* 17 (9), 1969–1983. doi:10.1007/s00784-013-1030-3

Martens, W., Wolfs, E., Struys, T., Politis, C., Bronckaers, A., and Lambrichts, I. (2012). Expression Pattern of Basal Markers in Human Dental Pulp Stem Cells and Tissue. *Cells Tissues Organs* 196 (6), 490–500. doi:10.1159/000338654

Martin, D. E., De Almeida, J. F. A., Henry, M. A., Khaing, Z. Z., Schmidt, C. E., Teixeira, F. B., et al. (2014). Concentration-Dependent Effect of Sodium Hypochlorite on Stem Cells of Apical Papilla Survival and Differentiation. *J. Endod.* 40 (1), 51–55. doi:10.1016/j.joen.2013.07.026

Martinez, V. G., Ontoria-Oviedo, I., Ricardo, C. P., Harding, S. E., Sacedon, R., Varas, A., et al. (2017). Overexpression of Hypoxia-Inducible Factor 1 Alpha Improves Immunomodulation by Dental Mesenchymal Stem Cells. *Stem Cell Res. Ther.* 8 (1), 208. doi:10.1186/s13287-017-0659-2

Martínez-Sarrà, E., Montori, S., Gil-Recio, C., Núñez-Toldrà, R., Costamagna, D., Rotini, A., et al. (2017). Human Dental Pulp Pluripotent-Like Stem Cells Promote Wound Healing and Muscle Regeneration. *Stem Cell Res. Ther.* 8 (1), 175. doi:10.1186/s13287-017-0621-3

Matoug-Elwerfelli, M., Nazzal, H., Raif, E. M., Wilshaw, S.-P., Esteves, F., and Duggal, M. (2020). *Ex-Vivo* Recellularisation and Stem Cell Differentiation of a Decellularised Rat Dental Pulp Matrix. *Sci. Rep.* 10 (1), 21553. doi:10.1038/s41598-020-78477-x

Mattei, V., Santacroce, C., Tasciotti, V., Martellucci, S., Santilli, F., Manganelli, V., et al. (2015). Role of Lipid Rafts in Neuronal Differentiation of Dental Pulp-Derived Stem Cells. *Exp. Cell Res.* 339 (2), 231–240. doi:10.1016/j.yexcr.2015.11.012

Melero-Martin, J. M., Khan, Z. A., Picard, A., Wu, X., Paruchuri, S., and Bischoff, J. (2007). *In Vivo* vasculogenic Potential of Human Blood-Derived Endothelial Progenitor Cells. *Blood* 109 (11), 4761–4768. doi:10.1182/blood-2006-12-062471

Miran, S., Mitsiadis, T. A., and Pagella, P. (2016). Innovative Dental Stem Cell-Based Research Approaches: The Future of Dentistry. *Stem Cell Int.* 2016, 1–7. doi:10.1155/2016/7231038

Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L. W., Robey, P. G., et al. (2003). SHED: Stem Cells from Human Exfoliated Deciduous Teeth. *Proc. Natl. Acad. Sci. U.S.A.* 100 (10), 5807–5812. doi:10.1073/pnas.0937635100

Morotomi, T., Washio, A., and Kitamura, C. (2019). Current and Future Options for Dental Pulp Therapy. *Jpn. Dental Sci. Rev.* 55 (1), 5–11. doi:10.1016/j.jdsr.2018.09.001

Mullen, S. F., and Critser, J. K. (2007). The Science of Cryobiology. *Cancer Treat. Res.* 138, 83–109. doi:10.1007/978-0-387-72293-1_7

Murakami, M., Hayashi, Y., Iohara, K., Osako, Y., Hirose, Y., and Nakashima, M. (2015). Trophic Effects and Regenerative Potential of Mobilized Mesenchymal Stem Cells from Bone Marrow and Adipose Tissue as Alternative Cell Sources

for Pulp/Dentin Regeneration. *Cell Transpl.* 24 (9), 1753–1765. doi:10.3727/096368914X683502

Nakamura, S., Yamada, Y., Katagiri, W., Sugito, T., Ito, K., and Ueda, M. (2009). Stem Cell Proliferation Pathways Comparison between Human Exfoliated Deciduous Teeth and Dental Pulp Stem Cells by Gene Expression Profile from Promising Dental Pulp. *J. Endod.* 35 (11), 1536–1542. doi:10.1016/j.joen.2009.07.024

Nakashima, M., Iohara, K., Murakami, M., Nakamura, H., Sato, Y., Ariji, Y., et al. (2017). Pulp Regeneration by Transplantation of Dental Pulp Stem Cells in Pulpitis: A Pilot Clinical Study. *Stem Cell Res. Ther.* 8 (1), 61. doi:10.1186/s13287-017-0506-5

Nakashima, M., Iohara, K., and Sugiyama, M. (2009). Human Dental Pulp Stem Cells with Highly Angiogenic and Neurogenic Potential for Possible Use in Pulp Regeneration. *Cytokine Growth Factor. Rev.* 20 (5–6), 435–440. doi:10.1016/j.cytogfr.2009.10.012

Nam, H., Kim, G.-H., Bae, Y.-K., Jeong, D.-E., Joo, K.-M., Lee, K., et al. (2017). Angiogenic Capacity of Dental Pulp Stem Cell Regulated by SDF-1α-CXCR4 Axis. *Stem Cell Int.* 2017, 1–10. doi:10.1155/2017/8085462

Nesti, C., Pardini, C., Barachini, S., D'Alessandro, D., Siciliano, G., Murri, L., et al. (2011). Human Dental Pulp Stem Cells Protect Mouse Dopaminergic Neurons Against MPP+ or Rotenone. *Brain Res.* 1367, 94–102. doi:10.1016/j.brainres.2010.09.042

Ng, Y.-L., Mann, V., and Gulabivala, K. (2008). Outcome of Secondary Root Canal Treatment: a Systematic Review of the Literature. *Int. Endod. J.* 41 (12), 1026–1046. doi:10.1111/j.1365-2591.2008.01484.x

Niyazi, M., Zibaii, M. I., Chavoshinezhad, S., Hamidabadi, H. G., Dargahi, L., Bojnordi, M. N., et al. (2020). Neurogenic Differentiation of Human Dental Pulp Stem Cells by Optogenetics Stimulation. *J. Chem. Neuroanat.* 109, 101821. doi:10.1016/j.jchemneu.2020.101821

Nosrat, A., Homayounfar, N., and Oloomi, K. (2012). Drawbacks and Unfavorable Outcomes of Regenerative Endodontic Treatments of Necrotic Immature Teeth: A Literature Review and Report of a Case. *J. Endod.* 38 (10), 1428–1434. doi:10.1016/j.joen.2012.06.025

Nosrat, I. V., Widenfalk, J., Olson, L., and Nosrat, C. A. (2001). Dental Pulp Cells Produce Neurotrophic Factors, Interact with Trigeminal Neurons *In Vitro*, and Rescue Motoneurons after Spinal Cord Injury. *Dev. Biol.* 238 (1), 120–132. doi:10.1006/dbio.2001.0400

Omi, M., Hata, M., Nakamura, N., Miyabe, M., Kobayashi, Y., Kamiya, H., et al. (2016). Transplantation of Dental Pulp Stem Cells Suppressed Inflammation in Sciatic Nerves by Promoting Macrophage Polarization Towards Anti-Inflammation Phenotypes and Ameliorated Diabetic Polyneuropathy. *J. Diabetes Investig.* 7 (4), 485–496. doi:10.1111/jdi.12452

Oshima, M., and Tsuji, T. (2014). Functional Tooth Regenerative Therapy: Tooth Tissue Regeneration and Whole-Tooth Replacement. *Odontology* 102 (2), 123–136. doi:10.1007/s10266-014-0168-z

Ostby, B. N. (1961). The Role of the Blood Clot in Endodontic Therapy. An Experimental Histologic Study. *Acta Odontol Scand.* 19, 324–353. doi:10.3109/00016356109043395

Özdemir, A. T., Özgül Özdemir, R. B., Kırmaz, C., Sarıboyacı, A. E., Ünal Halbutoğlları, Z. S., Özel, C., et al. (2016). The Paracrine Immunomodulatory Interactions between the Human Dental Pulp Derived Mesenchymal Stem Cells and CD4 T Cell Subsets. *Cell Immunol.* 310, 108–115. doi:10.1016/j.cellimm.2016.08.008

Pegg, D. E. (2015). Principles of Cryopreservation. *Methods Mol. Biol.* 1257, 3–19. doi:10.1007/978-1-4939-2193-5_1

Pierdomenico, L., Bonsi, L., Calvitti, M., Rondelli, D., Arpinati, M., Chirumbolo, G., et al. (2005). Multipotent Mesenchymal Stem Cells with Immunosuppressive Activity Can Be Easily Isolated from Dental Pulp. *Transplantation* 80 (6), 836–842. doi:10.1097/01.tp.0000173794.72151.88

Pilbauerova, N., Schmidt, J., Soukup, T., Koberova Ivancakova, R., and Suchanek, J. (2021). The Effects of Cryogenic Storage on Human Dental Pulp Stem Cells. *Int. J. Med. Sci.* 22 (9), 4432. doi:10.3390/ijms22094432

Pisciotta, A., Riccio, M., Carnevale, G., Lu, A., De Biasi, S., Gibellini, L., et al. (2015). Stem Cells Isolated from Human Dental Pulp and Amniotic Fluid Improve Skeletal Muscle Histopathology in Mdx/SCID Mice. *Stem Cell Res. Ther.* 6, 156. doi:10.1186/s13287-015-0141-y

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., et al. (1999). Multilineage Potential of Adult Human Mesenchymal Stem Cells. *Science* 284 (5411), 143–147. doi:10.1126/science.284.5411.143

Ratajczak, J., Bronckaers, A., Dillen, Y., Gervois, P., Vangansewinkel, T., Driesen, R. B., et al. (2016). The Neurovascular Properties of Dental Stem Cells and Their Importance in Dental Tissue Engineering. *Stem Cell Int.* 2016, 1–17. doi:10.1155/2016/9762871

Reeves, R., and Stanley, H. R. (1966). The Relationship of Bacterial Penetration and Pulpal Pathosis in Carious Teeth. *Oral Surg. Oral Med. Oral Pathol.* 22 (1), 59–65. doi:10.1016/0030-4220(66)90143-5

Ricucci, D., Loghin, S., and Siqueira, J. F., Jr. (2014). Correlation between Clinical and Histologic Pulp Diagnoses. *J. Endod.* 40 (12), 1932–1939. doi:10.1016/j.joen.2014.08.010

Ring, K. C., Murray, P. E., Namerow, K. N., Kuttler, S., and Garcia-Godoy, F. (2008). The Comparison of the Effect of Endodontic Irrigation on Cell Adherence to Root Canal Dentin. *J. Endod.* 34 (12), 1474–1479. doi:10.1016/j.joen.2008.09.001

Ruangsawasdi, N., Zehnder, M., Patcas, R., Ghayor, C., Siegenthaler, B., Gjoksi, B., et al. (2017). Effects of Stem Cell Factor on Cell Homing during Functional Pulp Regeneration in Human Immature Teeth. *Tissue Eng. Part A* 23 (3–4), 115–123. doi:10.1089/ten.TEA.2016.0227

Salehrabi, R., and Rotstein, I. (2004). Endodontic Treatment Outcomes in a Large Patient Population in the USA: An Epidemiological Study. *J. Endod.* 30 (12), 846–850. doi:10.1097/01.don.0000145031.04236.ca

Selwitz, R. H., Ismail, A. I., and Pitts, N. B. (2007). Dental Caries. *Lancet* 369 (9555), 51–59. doi:10.1016/S0140-6736(07)60031-2

Seo, B.-M., Miura, M., Gronthos, S., Mark Bartold, P., Batouli, S., Brahim, J., et al. (2004). Investigation of Multipotent Postnatal Stem Cells from Human Periodontal Ligament. *Lancet* 364 (9429), 149–155. doi:10.1016/S0140-6736(04)16627-0

Shah, N., Logani, A., Bhaskar, U., and Aggarwal, V. (2008). Efficacy of Revascularization to Induce Apexification/apexogensis in Infected, Nonvital, Immature Teeth: A Pilot Clinical Study. *J. Endod.* 34 (8), 919–925. doi:10.1016/j.joen.2008.05.001

Shang, F., Ming, L., Zhou, Z., Yu, Y., Sun, J., Ding, Y., et al. (2014). The Effect of Licochalcone A on Cell-Aggregates ECM Secretion and Osteogenic Differentiation During Bone Formation in Metaphyseal Defects in Ovariectomized Rats. *Biomaterials* 35 (9), 2789–2797. doi:10.1016/j.biomaterials.2013.12.061

Shi, S., Bartold, P., Miura, M., Seo, B., Robey, P., and Gronthos, S. (2005). The Efficacy of Mesenchymal Stem Cells to Regenerate and Repair Dental Structures. *Orthod. Craniofac. Res.* 8 (3), 191–199. doi:10.1111/j.1601-6343.2005.00331.x

Shi, S., and Gronthos, S. (2003). Perivascular Niche of Postnatal Mesenchymal Stem Cells in Human Bone Marrow and Dental Pulp. *J. Bone Miner Res.* 18 (4), 696–704. doi:10.1359/jbmr.2003.18.4.696

Shi, X., Mao, J., and Liu, Y. (2020). Pulp Stem Cells Derived from Human Permanent and Deciduous Teeth: Biological Characteristics and Therapeutic Applications. *Stem Cell Transl. Med.* 9 (4), 445–464. doi:10.1002/sctm.19-0398

Simon, S., Perard, M., Zanini, M., Smith, A. J., Charpentier, E., Djole, S. X., et al. (2013). Should Pulp Chamber Pulpotomy be Seen as a Permanent Treatment? Some Preliminary Thoughts. *Int. Endod. J.* 46 (1), 79–87. doi:10.1111/j.1365-2591.2012.02113.x

Skoglund, A., Tronstad, L., and Wallenius, K. (1978). A Microangiographic Study of Vascular Changes in Replanted and Autotransplanted Teeth of Young Dogs. *Oral Surg. Oral Med. Oral Pathol.* 45 (1), 17–28. doi:10.1016/0030-4220(78)90217-7

Song, J. S., Takimoto, K., Jeon, M., Vadakekalam, J., Ruparel, N. B., and Diogenes, A. (2017). Decellularized Human Dental Pulp as a Scaffold for Regenerative Endodontics. *J. Dent. Res.* 96 (6), 640–646. doi:10.1177/0022034517693606

Sonoyama, W., Liu, Y., Fang, D., Yamaza, T., Seo, B.-M., Zhang, C., et al. (2006). Mesenchymal Stem Cell-Mediated Functional Tooth Regeneration in Swine. *PLoS One* 1, e79. doi:10.1371/journal.pone.0000079

Sonoyama, W., Liu, Y., Yamaza, T., Tuan, R. S., Wang, S., Shi, S., et al. (2008). Characterization of the Apical Papilla and its Residing Stem Cells from Human Immature Permanent Teeth: a Pilot Study. *J. Endod.* 34 (2), 166–171. doi:10.1016/j.joen.2007.11.021

Spaggiari, G. M., Capobianco, A., Becchetti, S., Mingari, M. C., and Moretta, L. (2006). Mesenchymal Stem Cell-Natural Killer Cell Interactions: Evidence that Activated NK Cells are Capable of Killing MSCs, whereas MSCs Can Inhibit IL-2-induced NK-Cell Proliferation. *Blood* 107 (4), 1484–1490. doi:10.1182/blood-2005-07-2775

Stevens, A., Zuliani, T., Olejnik, C., LeRoy, H., Obriot, H., Kerr-Conte, J., et al. (2008). Human Dental Pulp Stem Cells Differentiate into Neural Crest-Derived Melanocytes and Have Label-Retaining and Sphere-Forming Abilities. *Stem Cell Dev.* 17 (6), 1175–1184. doi:10.1089/scd.2008.0012

Su, W.-T., Chiou, W.-L., Yu, H.-H., and Huang, T.-Y. (2016). Differentiation Potential of SHEDs Using Biomimetic Periosteum Containing Dexamethasone. *Mater. Sci. Eng. C* 58, 1036–1045. doi:10.1016/j.msec.2015.09.077

Sugiyama, M., Iohara, K., Wakita, H., Hattori, H., Ueda, M., Matsushita, K., et al. (2011). Dental Pulp-Derived CD31−/CD146− Side Population Stem/Progenitor Cells Enhance Recovery of Focal Cerebral Ischemia in Rats. *Tissue Eng. Part A* 17 (9–10), 1303–1311. doi:10.1089/ten.TEA.2010.0306

Sui, B.-D., Hu, C.-H., Zheng, C.-X., Shuai, Y., He, X.-N., Gao, P.-P., et al. (2017). Recipient Glycemic Micro-Environments Govern Therapeutic Effects of Mesenchymal Stem Cell Infusion on Osteopenia. *Theranostics* 7 (5), 1225–1244. doi:10.7150/thno.18181

Sui, B., Hu, C., Zhang, X., Zhao, P., He, T., Zhou, C., et al. (2016). Allogeneic Mesenchymal Stem Cell Therapy Promotes Osteoblastogenesis and Prevents Glucocorticoid-Induced Osteoporosis. *Stem Cell Transl Med* 5 (9), 1238–1246. doi:10.5966/sctm.2015-0347

Suzuki, T., Lee, C. H., Chen, M., Zhao, W., Fu, S. Y., Qi, J. J., et al. (2011). Induced Migration of Dental Pulp Stem Cells for *In Vivo* Pulp Regeneration. *J. Dent Res.* 90 (8), 1013–1018. doi:10.1177/0022034511408426

Taha, N. A., and Khazali, M. A. (2017). Partial Pulpotomy in Mature Permanent Teeth with Clinical Signs Indicative of Irreversible Pulpitis: A Randomized Clinical Trial. *J. Endod.* 43 (9), 1417–1421. doi:10.1016/j.joen.2017.03.033

Takeuchi, N., Hayashi, Y., Murakami, M., Alvarez, F., Horibe, H., Iohara, K., et al. (2015). Similar *In Vitro* Effects and Pulp Regeneration in Ectopic Tooth Transplantation by Basic Fibroblast Growth Factor and Granulocyte-colony Stimulating Factor. *Oral Dis.* 21 (1), 113–122. doi:10.1111/odi.12227

Telukuntla, K. S., Suncion, V. Y., Schulman, I. H., and Hare, J. M. (2013). The Advancing Field of Cell-Based Therapy: Insights and Lessons from Clinical Trials. *J. Am. Heart Assoc.* 2 (5), e000338. doi:10.1161/JAHA.113.000338

Torabinejad, M., Anderson, P., Bader, J., Brown, L. J., Chen, L. H., Goodacre, C. J., et al. (2007). Outcomes of Root Canal Treatment and Restoration, Implant-Supported Single Crowns, Fixed Partial Dentures, and Extraction Without Replacement: A Systematic Review. *J. Prosthet. Dent.* 98 (4), 285–311. doi:10.1016/S0022-3913(07)60102-4

Trounson, A., and McDonald, C. (2015). Stem Cell Therapies in Clinical Trials: Progress and Challenges. *Cell Stem Cell* 17 (1), 11–22. doi:10.1016/j.stem.2015.06.007

Tziafas, D., Smith, A. J., and Lesot, H. (2000). Designing New Treatment Strategies in Vital Pulp Therapy. *J. Dent.* 28 (2), 77–92. doi:10.1016/s0300-5712(99)00047-0

Uccelli, A., Moretta, L., and Pistoia, V. (2008). Mesenchymal Stem Cells in Health and Disease. *Nat. Rev. Immunol.* 8 (9), 726–736. doi:10.1038/nri2395

Van Nieuwenhuysen, J.-P., Aouar, M., and D'Hoore, W. (1994). Retreatment or Radiographic Monitoring in Endodontics. *Int. Endod. J.* 27 (2), 75–81. doi:10.1111/j.1365-2591.1994.tb00234.x

Volponi, A. A., Pang, Y., and Sharpe, P. T. (2010). Stem Cell-Based Biological Tooth Repair and Regeneration. *Trends Cell Biol.* 20 (12), 715–722. doi:10.1016/j.tcb.2010.09.012

Wang, Y., Zhao, Y., Jia, W., Yang, J., and Ge, L. (2013). Preliminary Study on Dental Pulp Stem Cell-Mediated Pulp Regeneration in Canine Immature Permanent Teeth. *J. Endod.* 39 (2), 195–201. doi:10.1016/j.joen.2012.10.002

Woods, E. J., Perry, B. C., Hockema, J. J., Larson, L., Zhou, D., and Goebel, W. S. (2009). Optimized Cryopreservation Method for Human Dental Pulp-Derived Stem Cells and Their Tissues of Origin for Banking and Clinical Use. *Cryobiology* 59 (2), 150–157. doi:10.1016/j.cryobiol.2009.06.005

Yan, M., Nada, O. A., Kluwe, L., Gosau, M., Smeets, R., and Friedrich, R. E. (2020). Expansion of Human Dental Pulp Cells *In Vitro* Under Different Cryopreservation Conditions. *In Vivo* 34 (5), 2363–2370. doi:10.21873/invivo.12049

Yang, J.-w., Zhang, Y.-f., Wan, C.-y., Sun, Z.-y., Nie, S., Jian, S.-j., et al. (2015). Autophagy in SDF-1α-Mediated DPSC Migration and Pulp Regeneration. *Biomaterials* 44, 11–23. doi:10.1016/j.biomaterials.2014.12.006

Yang, J., Yuan, G., and Chen, Z. (2016). Pulp Regeneration: Current Approaches and Future Challenges. *Front. Physiol.* 7, 58. doi:10.3389/fphys.2016.00058

Yao, S., Pan, F., Prpic, V., and Wise, G. E. (2008). Differentiation of Stem Cells in the Dental Follicle. *J. Dent Res.* 87 (8), 767–771. doi:10.1177/154405910808700801

Yi, Q., Liu, O., Yan, F., Lin, X., Diao, S., Wang, L., et al. (2017). Analysis of Senescence-Related Differentiation Potentials and Gene Expression Profiles in Human Dental Pulp Stem Cells. *Cells Tissues Organs* 203 (1), 1–11. doi:10.1159/000448026

Yokoi, T., Saito, M., Kiyono, T., Iseki, S., Kosaka, K., Nishida, E., et al. (2006). Establishment of Immortalized Dental Follicle Cells for Generating Periodontal Ligament *In Vivo*. *Cell Tissue Res.* 327 (2), 301–311. doi:10.1007/s00441-006-0257-6

Yokomise, H., Inui, K., Wada, H., Ueda, M., and Hitomi, S. (1996). Long-Term Cryopreservation Can Prevent Rejection of Canine Tracheal Allografts with Preservation of Graft Viability. *J. Thorac. Cardiovasc. Surg.* 111 (5), 930–934. doi:10.1016/s0022-5223(96)70366-5

Yoshiba, N., Edanami, N., Tohma, A., Takeuchi, R., Ohkura, N., Hosoya, A., et al. (2018). Detection of Bone Marrow-Derived Fibrocytes in Human Dental Pulp Repair. *Int. Endod. J.* 51 (11), 1187–1195. doi:10.1111/iej.12940

Zhang, J., Huang, X., Wang, H., Liu, X., Zhang, T., Wang, Y., et al. (2015). The Challenges and Promises of Allogeneic Mesenchymal Stem Cells for Use as a Cell-Based Therapy. *Stem Cell Res. Ther.* 6, 234. doi:10.1186/s13287-015-0240-9

Zhang, L. X., Shen, L. L., Ge, S. H., Wang, L. M., Yu, X. J., Xu, Q. C., et al. (2015). Systemic BMSC Homing in the Regeneration of Pulp-Like Tissue and the Enhancing Effect of Stromal Cell-Derived Factor-1 on BMSC Homing. *Int. J. Clin. Exp. Pathol.* 8 (9), 10261–10271.

Zhang, W., Walboomers, X. F., Shi, S., Fan, M., and Jansen, J. A. (2006). Multilineage Differentiation Potential of Stem Cells Derived from Human Dental Pulp after Cryopreservation. *Tissue Eng.* 12 (10), 2813–2823. doi:10.1089/ten.2006.12.2813

Zhang, X., Li, H., Sun, J., Luo, X., Yang, H., Xie, L., et al. (2017). Cell-Derived Micro-Environment Helps Dental Pulp Stem Cells Promote Dental Pulp Regeneration. *Cell Prolif.* 50 (5), e12361. doi:10.1111/cpr.12361

Zhang, X.-M., Ouyang, Y.-J., Yu, B.-Q., Li, W., Yu, M.-Y., et al. (2021). Therapeutic Potential of Dental Pulp Stem Cell Transplantation in a Rat Model of Alzheimer's Disease. *Neural Regen. Res.* 16 (5), 893–898. doi:10.4103/1673-5374.297088

Zhao, H., Feng, J., Seidel, K., Shi, S., Klein, O., Sharpe, P., et al. (2014). Secretion of Shh by a Neurovascular Bundle Niche Supports Mesenchymal Stem Cell Homeostasis in the Adult Mouse Incisor. *Cell Stem Cell* 14 (2), 160–173. doi:10.1016/j.stem.2013.12.013

Zhao, Y., Wang, L., Jin, Y., and Shi, S. (2012). Fas Ligand Regulates the Immunomodulatory Properties of Dental Pulp Stem Cells. *J. Dent Res.* 91 (10), 948–954. doi:10.1177/0022034512458690

Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., et al. (2001). Multilineage Cells from Human Adipose Tissue: Implications for Cell-Based Therapies. *Tissue Eng.* 7 (2), 211–228. doi:10.1089/107632701300062859

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# Fibroadipogenic Progenitors Regulate the Basal Proliferation of Satellite Cells and Homeostasis of Pharyngeal Muscles via HGF Secretion

*Eunhye Kim[1,2], Fang Wu[1], Danbi Lim[1], Christopher Zeuthen[1], Yiming Zhang[1], James Allen[1], Laura Muraine[3], Capucine Trollet[3], Katherine E. Vest[4] and Hyojung J. Choo[1]**

[1]Department of Cell Biology, School of Medicine, Emory University, Atlanta, GA, United States, [2]Laboratory of Molecular Diagnostics and Cell Biology, College of Veterinary Medicine, Gyeongsang National University, Jinju, South Korea, [3]Sorbonne Université, Inserm, Institut de Myologie, U974, Centre de Recherche en Myologie, Paris, France, [4]Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH, United States

Skeletal muscle stem cells, known as satellite cells (SCs), are quiescent in normal adult limb muscles. Injury stimulates SC proliferation, differentiation, and fusion to regenerate muscle structure. In pharyngeal muscles, which are critical for swallowing foods and liquids, SCs proliferate and fuse in the absence of injury. It is unknown what factors drive increased basal activity of pharyngeal SCs. Here, we determined how niche factors influence the status of pharyngeal versus limb SCs. *In vivo*, a subset of pharyngeal SCs present features of activated SCs, including large cell size and increased mitochondrial content. In this study, we discovered that the pharyngeal muscle contains high levels of active hepatocyte growth factor (HGF), which is known to activate SCs in mice and humans. We found that fibroadipogenic progenitors (FAPs) are the major cell type providing HGF and are thus responsible for basal proliferation of SCs in pharyngeal muscles. Lastly, we confirmed the critical role of FAPs for pharyngeal muscle function and maintenance. This study gives new insights to explain the distinctive SC activity of pharyngeal muscles.

**Keywords: skeletal muscle stem cells, satellite cells, pharyngeal muscle, hepatocyte growth factor, fiboradipogenic progenitors, macrophages**

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****Correspondence:***
*Hyojung J. Choo*
*hchoo2@emory.edu*

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## INTRODUCTION

The pharynx is a muscular passageway of the digestive and respiratory tracts, which extends from the nasal and oral cavity to the larynx and esophagus. The pharynx contains a group of skeletal muscles that play a critical role in many vital processes, such as swallowing, breathing, and speaking. Like other craniofacial muscles, pharyngeal muscles originate from non-segmented cranial mesoderm during vertebrate embryogenesis, while trunk and limb muscles are derived from somites (Mootoosamy and Dietrich, 2002; Noden and Francis-West, 2006). These distinctive embryonic origins are associated with unique transcriptional regulatory networks in myogenic progenitor cells. For example, limb muscle development is controlled by the PAX3 transcription factor, while PITX2/TBX1 controls craniofacial muscle development (Sambasivan et al., 2011a). However, both early muscle development pathways converge to a common myogenic program that requires the expression of myogenic regulatory factors, such as MYF5, MYOD, and myogenin (Kelly et al., 2004; Harel et al., 2009; Sambasivan et al., 2009). While mature craniofacial and limb/trunk muscles are histologically very similar, they are differentially susceptible to muscular dystrophies. For

example, extraocular muscles are typically spared in Duchenne muscular dystrophy (Khurana et al., 1995) but are preferentially affected by oculopharyngeal muscular dystrophy (OPMD), a late-onset genetic disorder characterized by progressive dysphagia and ptosis (Victor et al., 1962).

An important common feature of craniofacial and limb/trunk muscles is the presence of muscle-specific stem cells termed satellite cells (SCs). SCs are a heterogeneous population of progenitor cells underneath the basal lamina of muscle fibers (Mauro, 1961; Lepper et al., 2009; Sambasivan et al., 2011b) that are crucial for skeletal muscle regeneration (Montarras et al., 2005; Sambasivan et al., 2011b; Lepper et al., 2011; Murphy et al., 2011; Fry et al., 2015). Like most other adult stem cells, SCs are quiescent under basal physiological conditions. When activated by injury, increased load, or disease, SCs rapidly re-enter the $G_1$ phase of the cell cycle, proliferate as myoblasts, and progress along a defined differentiation program, leading to myogenesis (Shi and Garry, 2006). The properties of SCs during regeneration have been extensively investigated using easily accessible limb muscles. Less is known about craniofacial muscle SCs, but it has been shown that SCs from pharyngeal muscles (Randolph et al., 2015) and extraocular muscles (Stuelsatz et al., 2015) contain a population of activated SCs that chronically proliferate and differentiate into myofibers in the absence of muscle damage. The increased SC activity in craniofacial muscles raises the question of whether their unique biological and physiological properties are influenced by cell intrinsic factors or by their specialized microenvironment, known as the niche. Multiple studies have demonstrated that extracellular components like collagen (Baghdadi et al., 2018), diffusible cytokines, and growth factors released from neighboring cells, such as resident or infiltrating macrophages and fibroadipogenic progenitors (FAPs) (Evano and Tajbakhsh, 2018; Helmbacher and Stricker, 2020; Theret et al., 2021), have a major influence on SC activity in limb muscles (Vishwakarma et al., 2017). FAPs are resident mesenchymal stem cells in muscle interstitium and have been reported to be activated after tissue damage (Joe et al., 2010). FAPs have been shown to support SC differentiation *in vitro* and are required for long-term homeostatic maintenance of skeletal muscle (Heredia et al., 2013; Wosczyna et al., 2019; Uezumi et al., 2021). However, it is not clear yet whether FAPs have a role in distinct properties of craniofacial SCs. In fact, very few studies have investigated how the unique niche of craniofacial muscles affects SC activity (Formicola et al., 2014).

In this study, we compared SCs from pharyngeal and gastrocnemius muscles to understand the relative contribution of cell-intrinsic and environmental factors to the elevated basal proliferation of pharyngeal SCs. We demonstrate that a subset of pharyngeal SCs resembles activated SCs with larger cell sizes and increased mitochondrial content. Additionally, we show that pharyngeal muscles contain an active form of hepatocyte growth factor (HGF), a known SC activator (Allen et al., 1995; Walker et al., 2015). We find that FAPs and $CD206^+$ resident macrophages secrete HGF and FAPs provide HGF activating enzymes including tissue-type plasminogen activator (PLAT). Lastly, based on experiments performed in FAPs-ablated mice, we conclude that FAPs within the pharyngeal muscle are partially responsible for the active proliferation of pharyngeal SCs and are critical for pharyngeal muscle function. These studies provide insight into the unique properties of craniofacial SCs and the craniofacial muscle niche, which may explain the differential susceptibility of these muscles to muscular dystrophies.

# MATERIALS AND METHODS

## Mice

C57BL/6J mice (Jax000664), *Pax7* $Cre^{ERT2}$ mice (Jax017763), *Rosa* $^{tdTomato}$ (Jax007909), *Rosa-DTA* (Jax009669), *Pax3 Cre* (Jax005549), *Rosa*$^{mT/mG}$ (Jax007576), PDGFRα $Cre^{ERT}$ (Jax018280), and Cx3cr1 $Cre^{ERT2}$ (Jax020940) were purchased from Jackson Laboratories (Bar Harbor, ME). Mouse age and genotype information was used as noted in figure legends. Homozygous *Pax7* $Cre^{ERT2}$ male mice were crossed with homozygous *Rosa*$^{flox\text{-}stop\text{-}flox\text{-}tdTomato}$ (tdTomato) to obtain *Pax7* $Cre^{ERT2/+}$; *Rosa* $^{tdTomato/+}$ (*Pax7* $Cre^{ERT2}$*-tdTomato*) mice (Sambasivan et al., 2011b). To label satellite cells with red fluorescence (tdTomato), tamoxifen at 1 mg (Sigma-Aldrich, St. Louis, MO) per 10 g body weight was injected intraperitoneally once daily for 5 days. Immunostaining was used to determine the recombination efficiency in *Pax7* $Cre^{ERT2}$*-tdTomato* mice. $tdTomato^+$ cells from pharyngeal and gastrocnemius muscles were attached to a glass slide by cytospin and immunostained with anti-Pax7 antibody to calculate efficiency ($n$ = 3). Gastrocnemius muscles (GA) have 97.4 ± 2.3% recombination efficiency and pharyngeal (PH) muscles have 90.5 ± 6.6% efficiency (**Supplementary Figure S1**), which is consistent with a previous report (Randolph et al., 2015). Quantitative polymerase chain reaction (qPCR) was used to determine the recombination efficiency in *Pax7* $Cre^{ERT2}$*-DTA* and PDGFRα $Cre^{ERT}$-DTA mice. Experiments were performed in accordance with approved guidelines and ethical approval from Emory University's Institutional Animal Care and Use Committee [PROTO20170233 (Choo)] and in compliance with the National Institutes of Health.

## Muscle Tissue Preparation

Pharyngeal tissue dissection was performed as described previously with small modifications (Randolph et al., 2015). Briefly, histologic sections included pharyngeal tissues that extend from the soft palate caudally to the cranial aspects of the trachea and esophagus. Gastrocnemius muscles were used as control limb muscles. Muscle tissues were frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) and stored at −80°C. Cross-sections were prepared longitudinally to capture circular outside constrictor muscles, including cricopharyngeal muscles. Tissue cross-sections of 10 µm thickness were collected every 100 µm using a Leica CM1850 cryostat.

For 3- or 7-day injured muscle tissues, mice were anesthetized with isoflurane. The injury was induced in tibialis anterior (TA) muscles of anesthetized mice by injection of 25 µl of 1.2% $BaCl_2$ using a Hamilton syringe (Cat# 8425 Hamilton, Reno, NV). For analgesia, mice were injected subcutaneously with 1 mg/kg

buprenorphine 72-h sustained-release (SR) before muscle injury. Muscles were collected 3 or 7 days after injury.

Human muscle tissues were obtained from autopsy and biopsy. Tibialis anterior and cricopharyngeus muscles were dissected by autopsy from donated bodies within 24 h postmortem as a part of the Emory Body Donor Program. Human biopsies (quadriceps and cricopharyngeal muscle) were obtained from the Myobank-AFM, a tissue bank affiliated to EuroBioBank and with national authorization to distribute human tissues (authorization AC-2019-3502 from the French Ministry of Research) with consent from the patient. Control cricopharyngeal muscles were obtained during otolaryngological surgery after informed consent in accordance with the French legislation on ethical rules.

## Fluorescence-Activated Cell Sorting

To obtain purified satellite cells (SCs), primary cells were isolated as described previously with small modifications (Choo et al., 2016). Briefly, dissected pharyngeal and gastrocnemius muscles were minced and digested using 0.2% collagenase II (Gibco, Carlsbad, CA) and 2.5 U/ml Dispase II (Gibco, Carlsbad, CA) in Dulbecco's modified Eagle's medium (DMEM) at 37°C while shaking at 80 rpm for 90 min. Digested muscles were then rinsed with the same volume of Ham's F10 media (11550043, Thermo Fisher Scientific, Waltham, MA) containing 20% FBS (F0926, Sigma, St. Louis, MO) and 100 μg/ml penicillin/streptomycin (P/S) (15140122, Thermo Fisher Scientific, Waltham, MA). Mononucleated cells were collected using a 70 μm cell strainer (Thermo Fisher Scientific, Waltham, MA). To facilitate rapid isolation of pure pharyngeal and hind limb SCs, we used a lineage labeling strategy where Pax7 positive SCs are marked with red fluorescence, tdTomato, upon tamoxifen-mediated Cre recombinase activation. Fluorescence-activated cell sorting (FACS) was performed using a BD FACSAria II cell sorter (Becton-Dickinson, Franklin Lakes, NJ) at the Emory University School of Medicine Core Facility for Flow Cytometry. Analyses of flow cytometry data were performed using FACSDiva (BD version 8.0.1) and FCS Express 6 Flow. FACS-purified SCs were plated at 500 cells per well in a 48-well plate coated with Matrigel (354277; Corning Life Sciences, New York, NY) and cultured for 5 days in Ham's F10 media containing 20% FBS and 25 ng/ml basic fibroblast growth factor (100-18B, PeproTech, Cranbury, NJ).

To sort $CD206^+$ macrophages, mononuclated cells were isolated as described above and labeled with surface proteins including 1:400 CD31-PE (clone 390; eBiosciences, San Diego, CA), 1:400 CD45-PE-Cy7 (clone 30-F11; BD Biosciences, San Jose, CA), 1:100 CD11b-FITC (clone M1/70; BD Biosciences, Vancouver, Canada), and 1:100 CD206-APC (clone C068C2; BioLegend, San Diego, CA). $CD206^+$ macrophages were collected according to the following sorting criteria, $CD31^-$/$CD45^+$/$CD11b^+$/$CD206^+$ using a BD FACSAria II cell sorter (Becton-Dickinson, Franklin Lakes, NJ) at the Emory University School of Medicine Core Facility for Flow Cytometry. After sorting, cells were centrifuged at 900 g for 10 min at 4°C. Cell pellets were snap-frozen by liquid nitrogen and stored in an ultra-low freezer for gene expression analysis.

## Cytospin

To attach isolated satellite cells on glass slides for cell size analysis and immunostaining, we put cells on cytofunnels (5991040, Thermo Fisher Scientific, Waltham, MA), assembled them with slide glasses filter cards, and spun down at 1,300 rpm for 3 min (Cytospin 3, Shandon). After centrifugation, cells were fixed with 2% paraformaldehyde for 10 min and washed with PBS 3 times before immunostaining.

## *In Vivo* Cell Proliferation Assays by Flow Cytometry

To compare the proliferative abilities of SCs in pharyngeal and hindlimb muscles *in vivo*, Bromo-2′-deoxyuridine (BrdU) assays were performed. Three-month-old male mice were injected with 10 μg BrdU (Sigma-Aldrich, St. Louis, MO)/g body weight intraperitoneally every 12 h for 2 days before sacrifice. Muscles were dissected and digested as described above. To assess proliferation, isolated mononucleated cells from pharyngeal or gastrocnemius muscles were immunostained with the following antibodies: 1:400 CD31-PE (clone 390; eBiosciences, San Diego, CA), 1:400 CD45-PE (clone 30-F11; BD Biosciences, San Jose, CA), 1:4000 Sca-1-PE-Cy7 (clone D7; BD Biosciences, Vancouver, Canada), and 1:20 a7-integrin-APC (FAB3518A; R&D SYSTEMS, Minneapolis, MN). Subsequently, cells were labeled for BrdU using a BrdU flow kit (BD biosciences, Vancouver, Canada), and proliferating SCs and FAPs were collected according to the following sorting criteria, $CD31^-$/$CD45^-$/$Sca1^-$/$Intergrin7\alpha^+$/$BrdU^+$ and $CD31^-$/$CD45^-$/$Sca1^+$/$BrdU^+$, respectively using BD FACS LSR II or BD FACSymphony A3 flow cytometer (flow rate: 300–1,000 event/sec). Single cells were selected by FSC-A vs. FSC-H and then selected by SSC-A vs. SSC-H (**Supplementary Figure S2A**). Gating for SCs, FAPs, BrdU was drawn by FMO samples (**Supplementary Figures S2B,C**). Cells from gastrocnemius muscles were used as limb muscle controls.

## Culture of Myogenic Progenitor Cells

Mononucleated cells were isolated from the hindlimb muscles as described previously (Jansen and Pavlath, 2006). Briefly, dissected pharyngeal and gastrocnemius muscles were minced and digested using 0.1% Pronase (EMD Millipore, Billerica, MA) and 25 mM HEPES in Dulbecco's modified Eagle's medium (DMEM) at 37°C while magnetic bar-stirring at 150 rpm for 60 min. Digested muscles were pelleted by centrifugation (1,000 g for 3 min) then rinsed with the same volume of DMEM media containing 10% FBS (F0926, Sigma, St. Louis, MO) and 100 μg/ml penicillin/streptomycin (P/S) (15140122, Thermo Fisher Scientific, Waltham, MA) for trituration using 25 ml serological pipette. Mononucleated cells were collected using a 70 μm cell strainer (Thermo Fisher Scientific, Waltham, MA). Isolated satellite cells were cultured in Ham's F-10 (11550043, Thermo Fisher Scientific, Waltham, MA), 20% FBS, 5 ng/ml basic fibroblast growth factor (100-18B, PeproTech, Cranbury, NJ), and P/S on plates coated with Collagen I (A1064402, Gibco, Thermo Fisher Scientific, Waltham, MA) for 3 or 5 days inside an incubator with 5% $CO_2$ and atmospheric $O_2$ concentration

(−20%) (HERAcell VIOS 160i $CO_2$ incubator, Thermo Fisher Scientific, Waltham, MA). If necessary, we performed pre-plating to maintain pure MPC culture (Gharaibeh et al., 2008). Briefly, trypsinized MPCs were incubated with culture media in regular tissue culture dishes without collagen coating for 45 min to allow the settling of rapidly adhering cells. Then medium with non-adhering cells including MPCs, was moved to collagen-coated tissues culture dishes. Culture medium was exchanged every other day. MPCs with fewer than 10 passages were used for experiments.

## Fusion Index and Nuclear Number Analysis

For the fusion assay of freshly isolated SCs, sorted SCs were cultured for 10 days to induce spontaneous differentiation (Stuelsatz et al., 2015). For the fusion assay of MPCs, pharyngeal and gastrocnemius MPCs were seeded at low density ($5 \times 10^3$ cells/cm$^2$) on Collagen I-coated plates to prevent cell-cell contact and differentiated for 2 days. Then, we counted and seeded cells at high density ($7.5 \times 10^4$ cells/cm$^2$) to initiate prompt fusion and further differentiated them for additional 2 days. At the end of differentiation, cells were fixed in 2% formaldehyde in PBS for 10 min at room temperature and stained with Phalloidin-iFluor 594 (ab176757; Abcam, United Kingdom) for 30 min at room temperature. Nuclei were then stained with 4′,6-diamidino-2-phenylindole (DAPI), and cells were mounted with Vectashield (Vector Labs, Burlingame, CA). Myoblast fusion was quantified by counting the number of myonuclei in myotubes, which were defined as containing two or more nuclei. Fusion index was calculated as the percentage of myonuclear number relative to the total number of nuclei in the images. Diameters of each myotube were measured at three points (1/4, 2/4, and 3/4 of the length) of a myotube and averaged for each myotube. We collected 10 images from random fields of view for each line.

## MitoTracker Staining

Pharyngeal and gastrocnemius muscles were dissected from *Pax7 Cre$^{ERT}$-tdTomato* mice, digested into mononuclear cells, and sorted in culture media (20% FBS in F-10 media with 1% P/S) using flow cytometry. Isolated cells were incubated with 50 nM MitoTracker® Green FM (M-7514; Life Technologies, Grand Island, NY) at 37°C for 30 min. The cells were washed twice in 2% FBS in HBSS buffer and observed by fluorescence microscopy (Revolve Echo, A Bico company, San Diego, CA). All images were taken on a Revolve Echo widefield fluorescence microscope using a x20 objective (UPlanFL N, Olympus) and 5 MP CMOS Monochrome Camera. To quantify SCs with high or low MitoTracker Green signal, we used flow cytometry (BD FACS LSR II flow cytometry). First, we gated tdTomato expressing cells, then we confirmed tdTomato population was clustered as a population in parent FSC-A vs. SSC-A scatter plot. We examined the tdTomato$^+$ population's mean value of FITC to detect the intensity of MitoTracker Green using FACSDiva. High MitoTracker Green gate of pharyngeal tdTomato cells was determined by MitoTracker Green histogram of gastrocnemius tdTomato cells. Overlapped plots were generated by FCS Express 6 Flow.

## Pharyngeal Mononucleated Cell Isolation by Magnetic-Activated Cell Sorting

Mononucleated cells were isolated from pharyngeal muscles using 0.2% collagenase II (Gibco, Carlsbad, CA) and 2.5 U/ml Dispase II (Gibco, Carlsbad, CA) in Dulbecco's modified Eagle's medium (DMEM) at 37°C while shaking at 80 rpm for 90 min. Cells were incubated for 1 min with ammonium-chloride-potassium (ACK) buffer to lyse red blood cells. Cells were washed with 2% BSA in PBS and labeled with biotin-CD31 antibodies (130-101-955; Miltenyi Biotec, Auburn, CA) (**Supplementary Figure S3A**). CD31$^+$ cells were isolated with magnetic streptavidin-coated microbeads and a magnetic column. Magnetic bound CD31$^+$ cells were collected by flushing the magnetic column with 1 ml of PBS. Subsequently, CD31$^-$ cells (unbound cells from previous MACS) were labeled with Biotin-CD45 antibodies (130-101-952; Miltenyi Biotec), and CD45$^+$ cells with anti-biotin microbeads were purified. Magnetic bound CD45$^+$ cells were collected by flushing the magnetic column with 1 ml of PBS. Unbound cells from both purifications were defined as CD31$^-$/CD45$^-$ cells. Lastly, CD31$^-$/CD45$^-$ cells (unbounded cells from previous MACS) were labeled with anti-PDGFRα microbeads (130-101-547; Miltenyi Biotec) using a magnetic column. Magnetic bound PDGFRα$^+$ cells were collected by flushing the magnetic column with 1 ml of PBS. Bound cells from both purifications were defined as CD31$^-$/CD45$^-$/PDGFRα$^+$ cells. Unbound cells from previous purifications were defined as CD31$^-$/CD45$^-$/PDGFRα$^-$ cells. Cell pellets were snap-frozen by liquid nitrogen and stored in an ultra-low freezer for gene expression analysis.

## Culture of Fibroadipogenic Progenitors

CD31$^-$/CD45$^-$/PDGFRα$^+$ cells were collected by MACS as above and cultured in DMEM, 10% FBS, 2.5 ng/ml basic fibroblast growth factor, and P/S on Matrigel-coated plates inside an incubator with 5% $CO_2$ and atmospheric $O_2$ concentration (−20%) (HERAcell VIOS 160i $CO_2$ incubator, Thermo Fisher Scientific, Waltham, MA) until 70% confluency. Culture medium was exchanged every other day. FAPs passeged 2 or 3 times were used to obtain media for ELISA of HGF.

## ELISA of Hepatocyte Growth Factor

Media were collected from dishes of 70% confluent FAPs isolated from either gastrocnemius or pharyngeal muscles. FAPs number was counted by hemocytometer to normalize HGF concentration after media collection. Fresh FAP growth medium was used as blank. We followed the user instructions for murine pre-coated HGF ELISA kit (BGK08048; PeproTech, Rocky Hill, NJ) to measure HGF concentration in FAPs media.

## Gene Expression Analysis by Real-Time Quantitative Polymerase Chain Reaction

Pharyngeal and gastrocnemius muscles and sorted cells from both muscles were analyzed for the expression of related markers by quantitative reverse transcriptase PCR (qPCR). Total RNA from samples was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Isolated RNA

(250 ng) was reverse transcribed into complementary DNA (cDNA) using random hexamers and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and analyzed by real-time qPCR. Amplification of cDNA was performed using Power SYBR® Green Master Mix (Applied Biosystems, Waltham, MA) and 2.5 μM of each primer. All primer sequences are listed in **Supplementary Table S1**. PCR reactions were performed for 35 cycles under the following conditions: denaturation at 95°C for 15 s and annealing + extension at 60°C for 1 min. Quantitative levels for all genes were normalized to endogenous *Hprt* expression for mouse and *RPLP0* expression for human except the SCs depletion experiment (*Pax7* gene expressions were normalized to *Acta1* gene expression). Fold change of gene expression was determined using the ΔΔCt method (Livak and Schmittgen, 2001).

## Immunoblotting

Pharyngeal, gastrocnemius, and 3 day-injured tibialis anterior mouse muscles were homogenized by Dounce homogenizer with 500 μl of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100) with protease and phosphatase inhibitors (Roche, Laval, Canada) (Sisson et al., 2009). Muscle lysates were incubated with lysis buffer for 1 h and centrifuged at 5,000 g for 10 min to remove tissues debris. Mouse HGF standard protein (50038-MNAH) was purchased from Sino Biological United States. Supernatants were collected and separated on SDS-polyacrylamide gel electrophoresis gels (4%–15% Mini-Protean TGX Stain-free gel, 4568086, Bio-rad, Hercules, CA) and transferred to nitrocellulose membrane (Bio-rad, Hercules, CA). The membranes were blocked in 5% non-fat dry milk for 1 h and incubated with primary antibodies (**Supplementary Table S2**) against HGF and GAPDH overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies {Donkey anti-mouse IgG-HRP [Jackson ImmunoResearch (715-035-15)] or Goat anti-Rabbit IgG-HRP [Bio Rad (170-6515)]} diluted 1:5000 for 1 h. Protein bands were detected using an enhanced chemiluminescence substrate kit (Thermo Fisher Scientific, Waltham, MA), and band intensity was measured by ImageJ.

## Immunohistochemistry/ Immunofluorescence

Immunohistochemistry/immunofluorescence was performed as follows: cryosections were incubated with blocking buffer (5% goat serum, 5% donkey serum, 0.5% BSA, 0.25% Triton-X 100 in PBS) for 1 h and labeled with primary antibodies (**Supplementary Table S2**) or isotype controls overnight at 4°C in blocking buffer. The following day, sections were washed three times with washing buffer (0.2% Tween-20 in PBS) and incubated with fluorescence probe-conjugated secondary antibodies for 1 h at room temperature. We used mannose receptor-1 (CD206) as a marker for resident macrophages and platelet-derived growth factor receptor α (PDGFRα) as a marker for FAPs. The TSA Green kit (Tyramide Signal Amplification; Perkin Elmer, Waltham, MA) was used for CD206 and PFGFRα staining after 1 h of incubation with biotinylated donkey-anti-rabbit F(ab′)2 IgG fragments (2.5 μg/ml) to enhance the immunostaining signal. Nuclei were then stained with DAPI (1 μg/ml) and mounted using Vectashield (Vector Labs, Burlingame, CA). All images were taken on a Revolve Echo widefield fluorescence microscope using a x10 (PlanC N, Olympus) or a x20 objective (UPlanFL N, Olympus) and 5 MP CMOS Monochrome Camera.

## Behavior Measurement

Each mouse was transferred from its home cage into a new cage for single housing. We weighed a water bottle (a sipper tube with 50 ml conical tube) and dry food (Laboratory Rodent Diet 5001) and placed them in each cage. Water and food consumption and body weight for each mouse were measured at the same time every day for 4 days. On the 4th day, water was removed from the mice cage for 16 h (Overnight). After 16 h have elapsed, we reintroduced the water one at a time for each mouse and recorded the lick episodes about 30 s upon the reintroduction of water. Using the IMovie app, we changed the video play speed to 0.1X and counted the tongue protrusions/second for each mouse.

## Statistical Analyses

Statistical analysis was performed using Prism 9.0. Results are expressed as the mean ± standard error of the mean (SEM). Experiments were repeated at least three times unless a different number of repeats is stated in the legend. Statistical testing was performed using the unpaired student's t-test or ANOVA if the data was normaly distributed by passing normality test (Shapiro-Wilk test and/or Kolmogorov-Smirnov test). If data is not normally distributed, we used non-parametric test, such as Mann-Whitney test for comparing 2 samples and Kruskal-Wallis test for comparing three or more samples. $p < 0.05$ was considered statistically significant. Statistical method, *p*-values, and sample numbers are indicated in the figure legends.

# RESULTS

## A Subset of Pharyngeal Satellite Cells Presents Features of Activated Satellite Cells

We focused on laryngeal pharynx muscles, including thyropharyngeus (TP) and cricopharyngeus (CP) muscles (**Supplementary Figure S4A**), which are involved in several pharyngeal pathologies including cricopharyngeal spasm (Arenaz Búa et al., 2015) and oculopharyngeal muscular dystrophy (Gómez-Torres et al., 2012). Although pharyngeal muscles are not derived from $Pax3^+$ myogenic progenitor as limb muscles are (**Supplementary Figure S4B**), both craniofacial and limb satellite cells (SCs) are distinguished by the expression of the paired-box/homeodomain transcription factor, PAX7. PAX7 is expressed during quiescence and early activation stages of SCs (Bosnakovski et al., 2008) and plays a key role in maintenance of self-renewed SCs (Oustanina et al., 2004). To investigate the SCs in pharyngeal muscles, we used a genetically engineered, tamoxifen-inducible *Pax7* $Cre^{ERT2}$-

*tdTomato* mouse, which labels all PAX7 lineage-derived cells with red fluorescent protein (tdTomato) (**Supplementary Figure S1**). Ten days after tamoxifen injection, we observed tdTomato-labeled SCs in sectioned TP, CP, and gastrocnemius (GA, limb) muscles (**Supplementary Figure S4C**). The number of tdTomato-SCs in CP muscles was twice the number of SCs in GA and TP muscles (**Supplementary Figure S4D**), indicating that SC density is variable in different muscles (Keefe et al., 2015).

When quiescent SCs are activated and begin proliferating, cell sizes are enlarged due to increased cytosolic volume and mitochondrial contents to support the energy demands of the activation (Rodgers et al., 2014). To investigate whether pharyngeal SCs exhibit characteristics similar to quiescent SCs isolated from gastrocnemius muscles, we measured the area of pharyngeal and gastrocnemius tdTomato-labeled SCs concentrated onto glass slides by cytospin (**Figure 1A**). The pharyngeal SCs were approximately 35% larger, with a mean area of 68.61 ± 1.611 $\mu m^2$ compared to the gastrocnemius SC mean area of 50.89 ± 1.437 $\mu m^2$. Interestingly, we identified two populations of pharyngeal SC that varied by size; the majority of pharyngeal SCs were similar in size to gastrocnemius SCs, but 21.5% of pharyngeal SCs were twice that size at over 100 $\mu m^2$ (Above the dot line in **Figure 1A**). To confirm that the larger pharyngeal SC population represents proliferating SCs, we labeled proliferating cells using bromodeoxyuridine (BrdU). We found that $BrdU^+$ pharyngeal SCs exhibit higher forward side scatter (FSC-A), indicating a larger cell size (**Figures 1B,C**). In addition, pharyngeal SCs contain increased mitochondria content as detected by MitoTracker Green (MTG) staining (**Figure 1D**) (Poot and Pierce, 1999). Quantification of MTG staining using flow cytometry (**Figures 1E,F**) revealed that 12% of total pharyngeal SCs contained higher mitochondrial contents (**Figure 1G**). Those pharyngeal SC populations with increased MTG signal were larger (increased FSC-A values) as evidenced by the slightly shifted peak in **Figure 1H** and average of FSC-A values (**Figure 1I**) and had higher granularity (increased SSC-A values) than those with low MTG signal (**Figures 1J,K**). To evaluate the activation status of pharyngeal SCs, we performed immunostaining using anti-MyoD antibodies on pharyngeal muscle sections of *Pax7* $Cre^{ERT2}$*-tdTomato* mouse (**Supplementary Figure S4E**). However, we did not find any $MyoD^+$ SCs on pharyngeal muscle sections, which is consistent with previous microarray data indicating that pharyngeal SCs express low levels of myogenic regulatory factors including *MyoD*, *Myf5*, and *myogenin* (Randolph et al., 2015). Taken together, a subset of pharyngeal SCs is proliferating as well as presenting the features of activated SCs, such as larger cell size and increased mitochondria contents, without expression of a known SC activation marker, such as MyoD.

## Extrinsic Factors Mediate Elevated Pharyngeal Satellite Cell Proliferation

To determine whether this proliferative activity is an intrinsic property of pharyngeal SCs or is due to the pharyngeal muscle microenvironment *in vivo*, we isolated pharyngeal SCs and measured proliferation *in vitro*. SCs were sorted by tdTomato fluorescence from pharyngeal and gastrocnemius muscles of *Pax7* $Cre^{ERT2}$*-tdTomato* mice (**Figure 2A**). Equal numbers of freshly-sorted SCs from pharyngeal and gastrocnemius were seeded and then cultured for 5 days (**Figure 2B**). After 5 days of culture, we counted the number of cells and found twice the number of pharyngeal SCs relative to the number of gastrocnemius SCs (**Figure 2C**). To investigate the differentiation potential of pharyngeal SCs, we cultured freshly-sorted satellite cells for 10 days to induce spontaneous differentiation (Stuelsatz et al., 2015). The cultured pharyngeal SCs consistently exhibited increased fusion at day 10 (**Figure 2D**), along with an increased fusion index (**Figure 2E**). However, the diameter of myotubes differentiated from pharyngeal SC was similar to the myotubes from gastrocnemius SCs (**Figure 2F**). These results indicate that freshly isolated pharyngeal SCs still retain highly proliferative and differentiative properties *in vitro* compared to the limb satellite cells.

Although *in vitro* pure SC culture experiments do not contain niche factors, prior exposure to niche factors *in vivo* may impact *ex vivo* proliferation and differentiation of SC in culture. For example, both activated SC and SC in the G-alert state, which are primed for activation by distant muscle injury (Rodgers et al., 2017), exhibit shorter first division time in the culture than freshly isolated quiescent SCs (Rodgers et al., 2014). To test whether higher proliferation and differentiation capacity found in pharyngeal SCs are intrinsic properties, we minimized the influence of prior niche factor exposure by studying pharyngeal and gastrocnemius-derived myogenic progenitor cells (MPCs) in culture. MPCs are established by growing SCs for a minimum of three passages and are more lineage-progressed than freshly isolated SCs. We seeded equal numbers of pharyngeal or gastrocnemius MPCs and cultured them for 2 days in identical conditions before performing a BrdU proliferation assay. As shown in **Figure 3G**, pharyngeal and gastrocnemius MPCs showed a similar level of $BrdU^+$ proliferating cells. To assess differentiation capacity, we used a two-step differentiation protocol to minimize the effect of any differences in proliferation (Girardi et al., 2021). Pharyngeal and gastrocnemius MPCs were seeded at low density to prevent cell-cell contact and differentiated for 2 days (**Figure 2H**, top). After 48 h cells were re-seeded at high density for an additional 2 days to initiate prompt fusion (**Figure 2H**, bottom). We confirmed that the fusion index for pharyngeal MPCs was not significantly different from the gastrocnemius MPCs (**Figure 2I**). Taken together, these results suggest that the high levels of proliferation and differentiation in pharyngeal SCs are only observed in *in vivo* (Randolph et al., 2015) or in freshly isolated pharyngeal SCs *in vitro*. Therefore, although we can not exclude intrinsic factors, our data suggest that the high levels of pharyngeal SC proliferation and differentiation could be the result of the extrinsic factors in pharyngeal muscles.

## Fibroadipogenic Progenitors and Resident Macrophages are Responsible for the High Level of Hepatocyte Growth Factor Transcript in Pharyngeal Muscles

Hepatocyte growth factor (HGF) is a well-known activator of quiescent SCs upon muscle injury (Allen et al., 1995; Walker et al., 2015), so we hypothesized that HGF, which could be one of extrinsic factors, contributes to proliferation of pharyngeal SC *in vivo*. To demonstrate if HGF plays a role in a proliferating subset

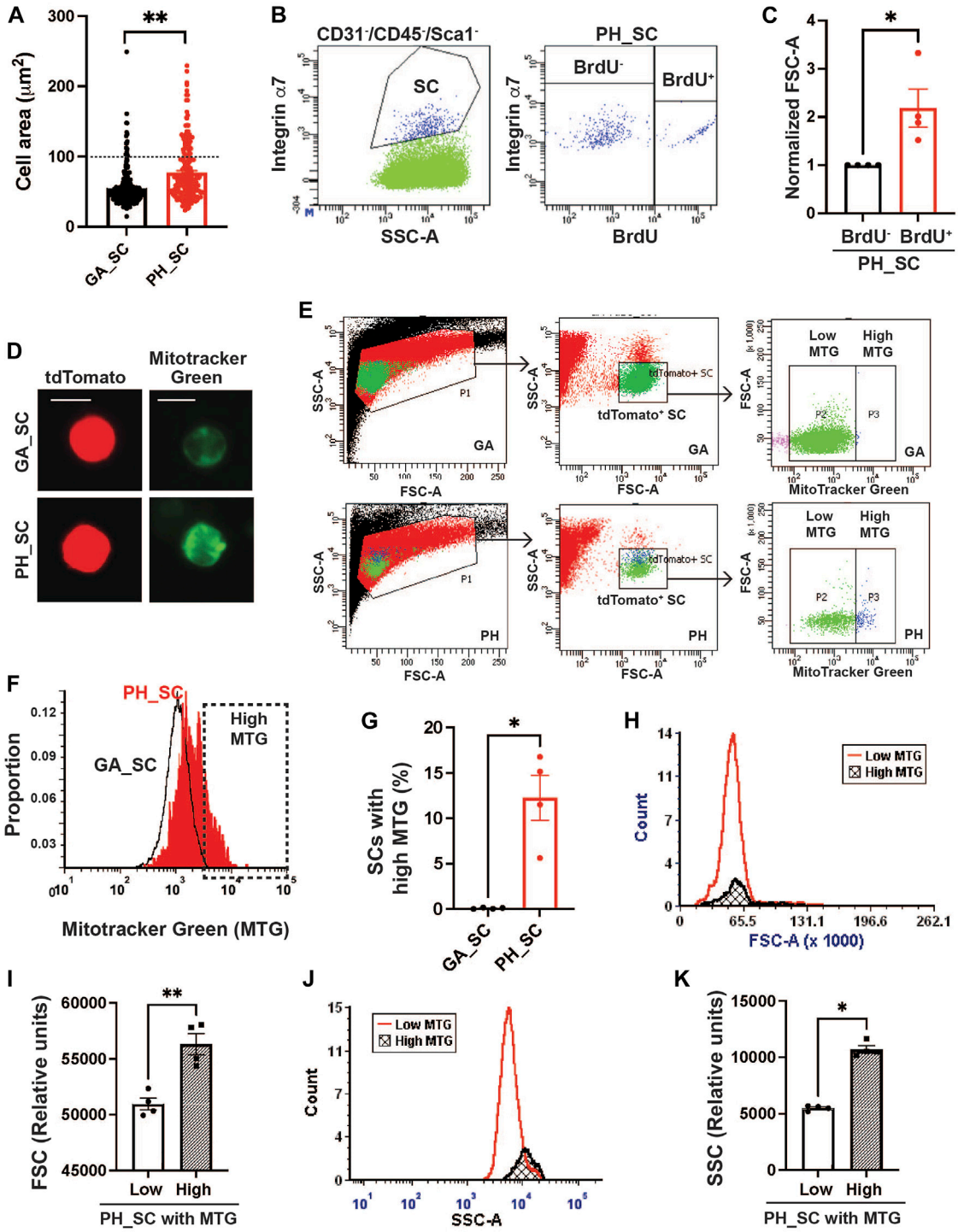


**FIGURE 1 |** A subset of pharyngeal satellite cells are enlarged and contain abundant mitochondria. **(A)** Measured cell area (μm$^2$) of pharyngeal (PH_SC) ($n$ = 3, 219 cells were analyzed) and gastrocnemius SCs (GA_SC) ($n$ = 3, 268 cells were analyzed) of 3 months old *Pax7 CRE$^{ERT2}$-tdtomato* mice using cytospin. **(B)** Representative scatter plot showing gating strategy for pharyngeal satellite cells (SC) (Left) and BrdU$^+$ pharyngeal satellite cells (Right). Satellite cells were isolated from 3 months old C57/BL6 mice. **(C)** Comparison of forward scatter (FSC-A) values between BrdU$^+$ pharyngeal SC and BrdU$^-$ pharyngeal SCs. FSC-A values were normalized to BrdU$^-$ pharyngeal SC's FSC. **(D)** Microscopic fluorescence images showing tdToamto expressing SCs from gastrocnemius or pharyngeal muscles mitochondria stained with MitoTracker Green. Scale bars = 10 μm. **(E)** Represntative flow gating for tdTomato$^+$ GA SC and PH SC (green dots) with high (blue dots) and low MitoTracker Green intensity. **(F)** Representative flow cytometry histogram of MitoTracker Green fluorescence levels of both gastrocnemius (black line) and pharyngeal (red line) SCs. The High MTG gate indicates the SC population with high levels of MitoTracker Green. Satellite cells were isolated from 3 months Pax7CRE$^{ERT2}$-tdtomato

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**FIGURE 1 |** mice. **(G)** Percentage of SCs with high levels of MitoTracker Green (MTG) in pharyngeal (PH_SC) and gastrocnemius SCs (GA_SC). $n = 4$. **(H, J)** Representative flow cytometry histogram of forward scatter (FSC-A) or side scatter (SSC-A) comparing pharyngeal SCs (PH_SC) with low and high MitoTracker Green (MTG). **(I, K)** Quantified forward scatter (FSC-A) or side scatter (SSC-A) values for pharyngeal SCs (PH_SC) with low and high MitoTracker Green (MTG) as determined by flow cytometry. $n = 4$. Statistical significance was determined by Mann-Whitney test **(C, G, K)** or by Student's t-test **(I)**. For all graphs, the value represents mean ± SEM. Asterisks indicate statistical significance ($*p < 0.05$, $**p < 0.01$).

of SC in pharyngeal muscles, we studied the levels of *Hgf* transcript. We confirmed that the level of *Hgf* transcript is significantly increased in pharyngeal muscles compared to gastrocnemius muscles (**Figure 3A**). To identify the HGF secreting cells in pharyngeal muscles, we isolated pharyngeal myofibers, as well as mononucleated cells that reside in pharyngeal muscles. The mononucleated cells were further sorted into endothelial cells ($CD31^+$), differentiated hematopoietic cells including macrophages ($CD31^-/CD45^+$), FAPs ($CD31^-/CD45^-/PDGFR\alpha^+$) (Motohashi et al., 2008), and other cell types including SCs ($CD31^-/CD45^-/PDGFR\alpha^-$), using MACS (**Supplementary Figure S3A**). We confirmed that each sorted population has high purity by probing for marker genes for each population using qPCR (**Supplementary Figure S3B**). The $CD31^-/CD45^-/PDGFR\alpha^+$ FAPs from pharyngeal muscles contained significantly higher levels of *Hgf* mRNA compared to other cell types (**Figure 3B**). We next investigated the number of FAPs in pharyngeal muscles by immunostaining for PDGFRα (**Figure 3C**). We detected a significant increase in $PDGFR\alpha^+$ cells per 100 fibers in pharyngeal muscles relative to uninjured gastrocnemius muscles, but this number was only 75% of the number of $PDGFR\alpha^+$ cells detected in 7-day injured TA muscles (**Figure 3D**). Using flow cytometry, we confirmed that pharyngeal FAPs ($CD31^-/CD45^-/Sca1^+$) represent about 10% of mononucleated cells in pharyngeal muscles, which is 3 times higher than gastrocnemius FAPs content (**Figure 3E**). In addition, FAP proliferation ($CD31^-/CD45^-/Sca1^+/BrdU^+$) in pharyngeal muscles is 6 times higher than gastocenmius FAPs basal proliferation (**Figures 3F,G**). Taken together these results suggest that elevated HGF in pharyngeal muscles may be provided by the high numbers of FAPs within the niche.

We found *Hgf* RNA was also detected in the immune cell population ($CD31^-/CD45^+$) of uninjured pharyngeal muscles (**Figure 3B**). During muscle regeneration, anti-inflammatory M2 macrophages secrete HGF (Sisson et al., 2009) and HGF also promotes M2 macrophage polarity in regenerating muscles (Choi et al., 2019). Due to the absence of injury in pharyngeal muscles such as low level of infiltrating monocyte-derived $Cx3cr1^+$macrophages (Arnold et al., 2007; Wang et al., 2020) in pharyngeal muscles similar to one in gastrocnemius muscles (**Supplementary Figure S5**), we focused tissue-resident macrophages in skeletal muscles rather than injury-induced, infiltrating macrophages (Wang et al., 2020; Dick et al., 2022). We used CD206 as a marker of muscle-resident macrophages (Wang et al., 2020) and found that sorted resident macrophages ($CD31^-/CD45^+/CD11b^+/CD206^+$) expressed higher levels of *Hgf* transcript compared to the total immune cell population ($CD31^-/CD45^+$) (**Figure 3H**). Lastly, we performed immunostaining to count $CD206^+$ resident macrophages in pharyngeal sections (**Figure 3I**). The number of $CD206^+$ cells per 100 fibers in pharyngeal muscles was 7 times higher than in uninjured gastrocnemius but was only 50% of the number of $CD206^+$ cells in 7-day injured TA muscles (**Figure 3J**). These results indicate that pharyngeal muscles contain more FAPs and $CD206^+$ macrophages than uninjured gastrocnemius muscles but less than regenerating limb muscles.

Although the $CD31^-/CD45^-/PDGFR\alpha^-$ SC-containing populations of pharyngeal muscles exhibit a trend of increased levels of *Hgf* mRNA relative to pharyngeal myofibers ($p = 0.06$), we found HGF transcript levels are comparable between pharyngeal and limb SCs by microarray (Randolph et al., 2015). In addition, there was no significant difference in the levels of *Hgf* mRNA between *SC*-ablated [*Pax7* $Cre^{+/-}$-$DTA^{+/+}$ TM (Tamoxifen injected)] and control [*Pax7* $Cre^{+/-}$-$DTA^{+/+}$ corn oil (CO, vehicle injected)] pharyngeal muscles (**Supplementary Figure S6B**). This result suggests that SCs are not a major source of elevated *Hgf* mRNA detected in pharyngeal muscles. Taken together, our data suggest that the increased number of FAPs and $CD206^+$ macrophages are responsible for the high level of *Hgf* mRNA in pharyngeal muscles.

## Human Pharyngeal Muscles and Mouse Extraocular Muscles Contain High Levels of Hepatocyte Growth Factor mRNA and Contain Fibroadipogenic Progenitors and CD206+ Macrophages

In mouse cricopharyngeal muscles, FAPs and residential macrophage numbers are much higher than those in gastrocnemius muscles (**Figures 3D,J**). To confirm that the high levels of *Hgf* RNA and relatively high numbers of FAPs and macrophages we detected in pharyngeal muscles are not exclusive to mice, we compared human pharyngeal and limb muscles. We found that human CP muscles also contain increased levels of *HGF* and *PDGFRα* mRNA (**Figure 4A**), increased $CD90^+$ FAPs (**Figure 4B**) and $CD206^+$ macrophages (**Figures 4C,D**) compared to human limb muscles. These data suggest that human pharyngeal muscles, similar to mice, provide a unique niche that could contribute to increased SC number of human cricopharyngeal muscles (Gidaro et al., 2013). Thus, the specific SC-activating niche features we detected in murine pharyngeal muscles including elevated *Hgf* provided by FAPs and resident macrophages are also true in humans. Further studies should decipher whether these features could explain pharyngeal muscle-specific pathology in humans.

Like pharyngeal muscles, mouse extraocular SCs exhibit high levels of proliferation and differentiation *in vivo* (McLoon and Wirtschafter, 2002) and *in vitro* (Stuelsatz et al., 2015). To determine if HGF provided by FAPs and $CD206^+$

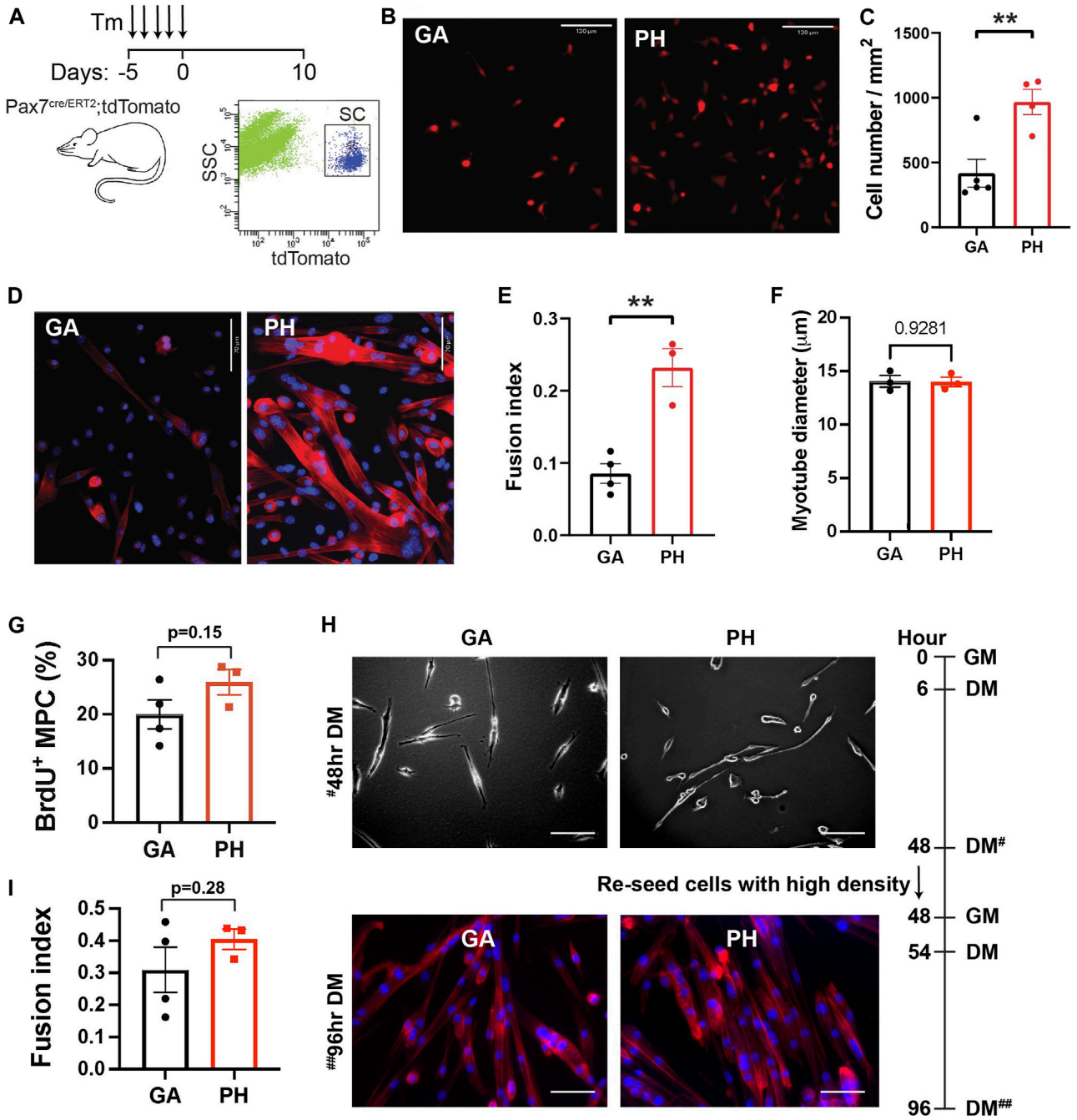


**FIGURE 2 |** High level of *in vitro* proliferation and differentiation of freshly isolated pharyngeal satellite cells but not pharyngeal myogenic progenitor cells. **(A)** Scheme of flow cytometry gating strategy for SC isolation using 3 months old *Pax7 Cre$^{ERT2}$-tdTomato* mice. **(B)** Representative image of 5-day cultured satellite cells derived from the gastrocnemius (GA) and pharyngeal (PH) muscles of 3 months *Pax7 Cre$^{ERT2}$-tdTomato* mice. Scale bars = 130 μm. **(C)** Analysis of cell number/mm$^2$ in 5-day cultured gastrocnemius and pharyngeal satellite cells. $n$ = 4–5. **(D)** Representative Phalloidin stained image (red) of 10-day cultured satellite cells derived from the gastrocnemius and pharyngeal muscles of 3 months *Pax7 Cre$^{ERT2}$-tdTomato* mice. Scale bars = 70 μm. The nucleus was stained by DAPI (blue). **(E)** Quantified fusion index at 10 days after culture. Fusion index was calculated as the percentage of total nuclei that resided in cells containing two or more nuclei. $n$ = 3–4. **(F)** The diameter of myotube at 10 days after culture. $n$ = 3, total of 148 GA myotubes and 353 PH myotubes were analyzed. **(G)** Percentage of BrdU$^+$ myogenic progenitor cells (MPCs) in gastrocnemius and pharyngeal muscles of 3 months C57BL/6 old mice. $n$ = 3. **(H)** Representative image of differentiated gastrocnemius (GA) and pharyngeal (PH) MPCs of 3 months C57BL/6 old mice. Growth medium (GM) was changed to differentiation medium (DM) 6 h after seeding. After first seeding, cells were re-seeded with high cell density in DM at 48 h$^{\#}$ with GM, and medium was changed to DM at 54 h. Scale bars = 130 μm. **(I)** Quantified fusion index at 96 h$^{\#\#}$. Fusion index was calculated as the percentage of total nuclei that resided in cells containing two or more nuclei. $n$ = 3, total of 264 GA myotubes and 282 PH myotubes were analyzed. Statistical significance was determined by Student's t-test **(C, E, G, I)** or by Mann-Whitney test **(F)**. For all graphs, the value represents mean ± SEM. Asterisks indicate statistical significance (**$p$ < 0.01).

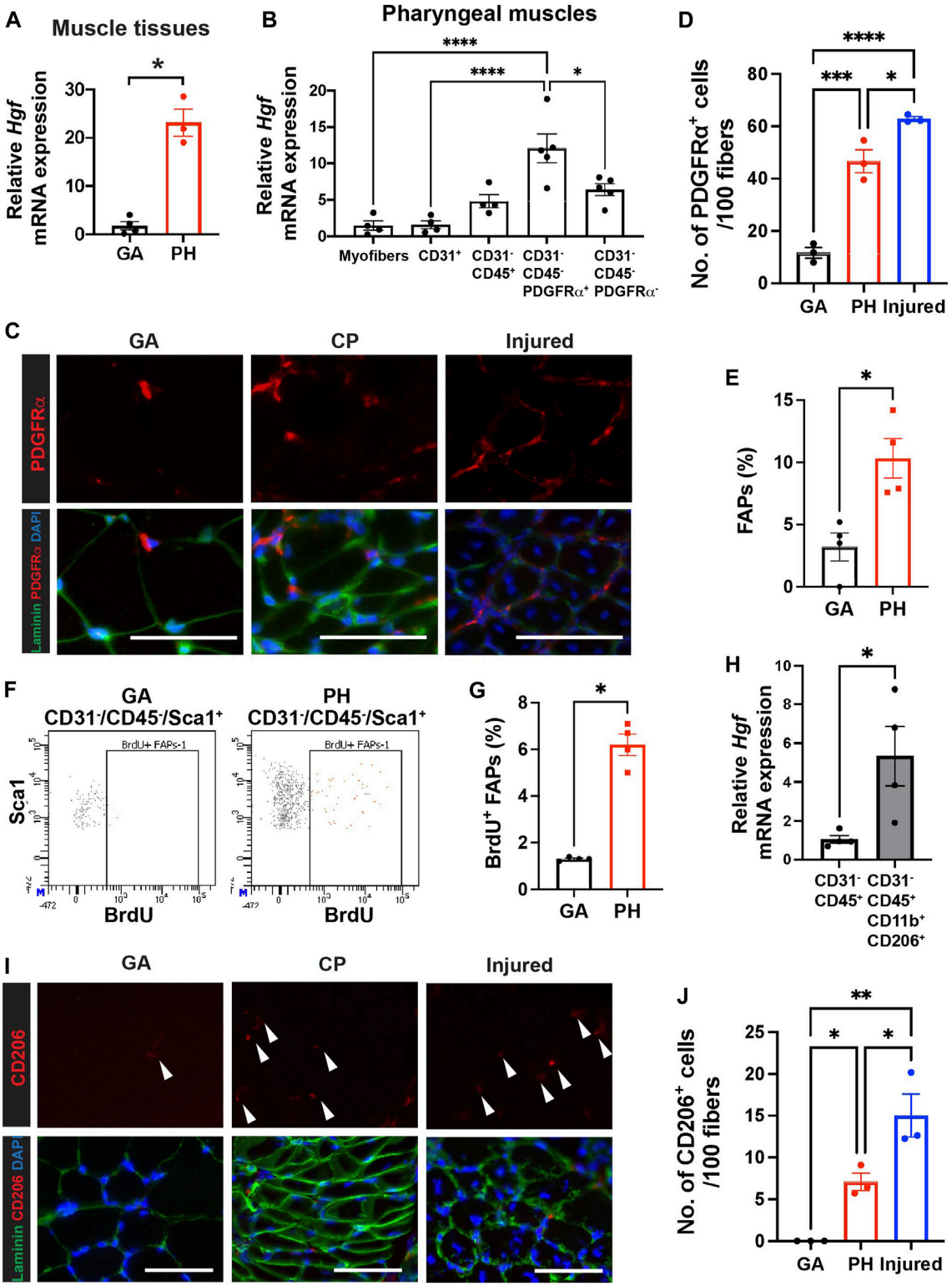


**FIGURE 3 |** Pharyngeal muscles contain high level of HGF, FAPs and CD206$^+$ macrophages. **(A)** Relative mRNA expression level of hepatocyte growth factor (*Hgf*) in gastrocnemius (GA) and pharyngeal (PH) muscles obtained from 3 month old C57BL/6 mice. $n$ = 3–4. **(B)** Relative mRNA expression level of *Hgf* in myofibers and MACS-sorted CD31$^+$, CD31$^-$/CD45$^+$, CD31$^-$/CD45$^-$/PDGFRα$^+$ and CD31$^-$/CD45$^-$/PDGFRα$^-$ cells obtained from muscles of 3 months old C57BL/6 mice. $n$ = 4 or 5. **(C)** Representative images of PDGFRα$^+$ cells in gastrocnemius (GA) and pharyngeal (PH) muscles of 3 months old C57BL/6 mice. Merged images show immunostaining with anti-PDGFRα (Red) and anti-laminin (green) antibodies and DAPI (blue). Scale bars = 50 μm. **(D)** Quantified number of PDGFRα$^+$ cells per 100

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**FIGURE 3 |** myofibers. 7-day $BaCl_2$-injured tibialis anterior (TA) muscles are used as a positive control. $n = 3$. **(E)** Proportion of FAPs ($CD31^-/CD45^-/Sca1^+$) population in mononucleated cells of gastrocnemius (GA) and pharyngeal (PH) muscles by flow cytometry. **(F)** Representative dot plots of $BrdU^+$ FAPs from gastrocnemius (GA) and pharyngeal (PH) muscles. **(G)** Quantified $BrdU^+$ FAPs from gastrocnemius (GA) and pharyngeal (PH) muscles by flow cytometry. **(H)** The relative mRNA expression level of hepatocyte growth factor (*Hgf*) in MACS-sorted immune cells ($CD31^-/CD45^+$) and FACS-sorted $CD206^+$ macrophages ($CD31^-/CD45^+/CD11b^+/CD206^+$) from pharyngeal muscles of 3 months old C57BL/6 mice. **(I)** Representative images of $CD206^+$ cells in gastrocnemius (GA) and pharyngeal (PH) muscles of 3 months old C57/BL6 mice. Merged images show immunostaining with anti-CD206 (Red) and anti-laminin (green) antibodies and DAPI (blue). Scale bars = 50 μm. **(J)** A quantified number of $CD206^+$ cells per 100 myofibers. 7-day $BaCl_2$-injured tibialis anterior (TA) muscles are used as a positive control $n = 3$. Statistical significance was determined by student's t-test **(A, E)**, by Mann-Whitney test **(G, H)**, or by 1-way ANOVA **(B, D, J)**. The value represents mean ± SEM. Asterisks indicate statistical significance (*$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

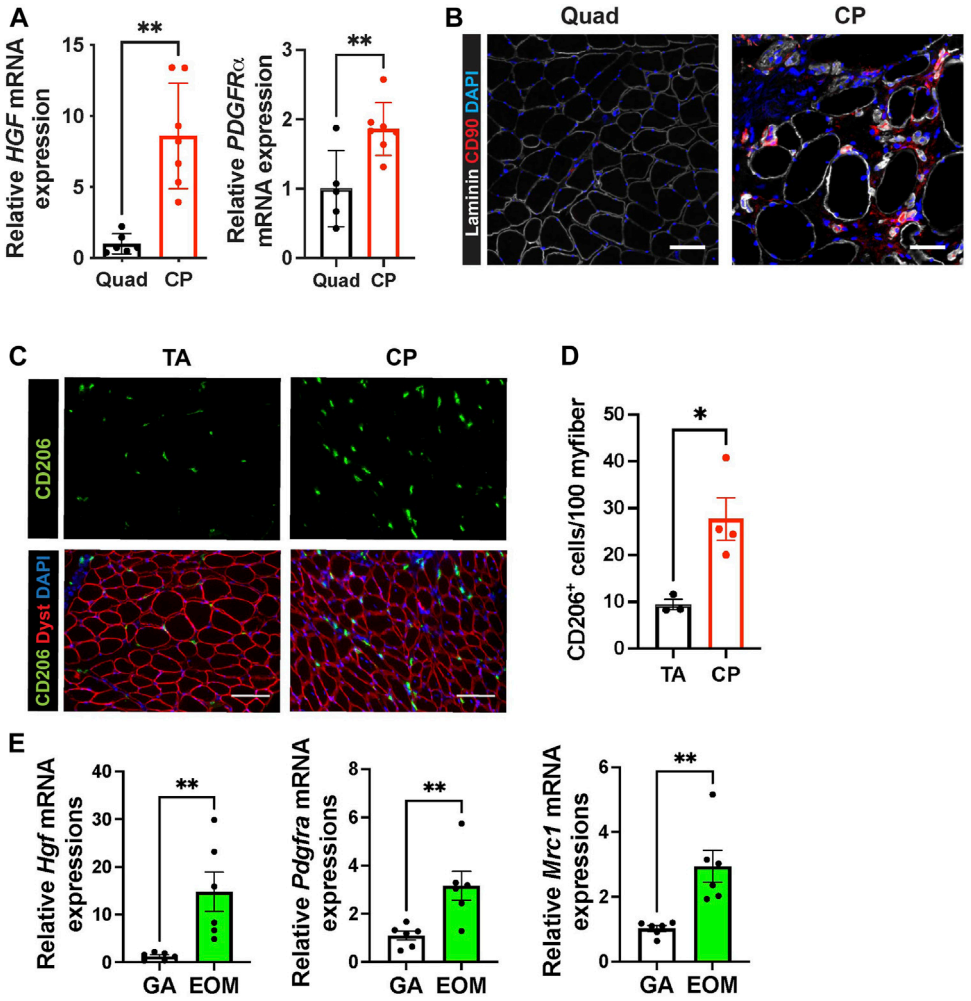


**FIGURE 4 |** Human pharyngeal muscles and mouse extraocular muscles contain an increased level of hepatocyte growth factor, FAPs, and $CD206^+$ macrophages. **(A)** Relative mRNA expression level of *HGF* and *PDGFRa* in human quadriceps (Quad) and cricopharyngeus (CP) muscles. $n = 5–7$. **(B)** Representative images of $CD90^+$ FAP cells in human quadriceps (Quad) and cricopharyngeus (CP) muscles. Merged images show immunostaining with anti-CD90 (red) and anti-laminin (white) antibodies and DAPI (blue). Scale bars = 50 μm. **(C)** Representative images of $CD206^+$ cells in human tibialis anterior (TA) and cricopharyngeus (CP) muscles. Merged images show immunostaining with anti-CD206 (green) and anti-dystrophin (red) antibodies and DAPI (blue). Scale bars = 130 μm. **(D)** The quantified number of $CD206^+$ cells per 100 myofibers. $n = 3–4$. **(D)** Relative mRNA expression level of *Hgf*, *Pdgfra*, *Mrc1* in mouse gastrocnemius (GA) and extraocular muscles (EOM) $n = 6$. Statistical significance was determined by student's t-test **(A, D, E)**. The value represents mean ± SEM. Asterisks indicate statistical significance (*$p < 0.05$ and **$p < 0.01$).

macrophages contributes to the proliferation and differentiation of SCs in extraocular muscles, we interrogated mouse extraocular muscles. We detected high levels of *Hgf* mRNA and markers for FAPs (*Pdgfra*) and $CD206^+$ macrophages (*Mrc1*) in mouse extraocular muscles (**Figure 4E**). Taken together, these data suggest that elevated levels of HGF could be a common factor

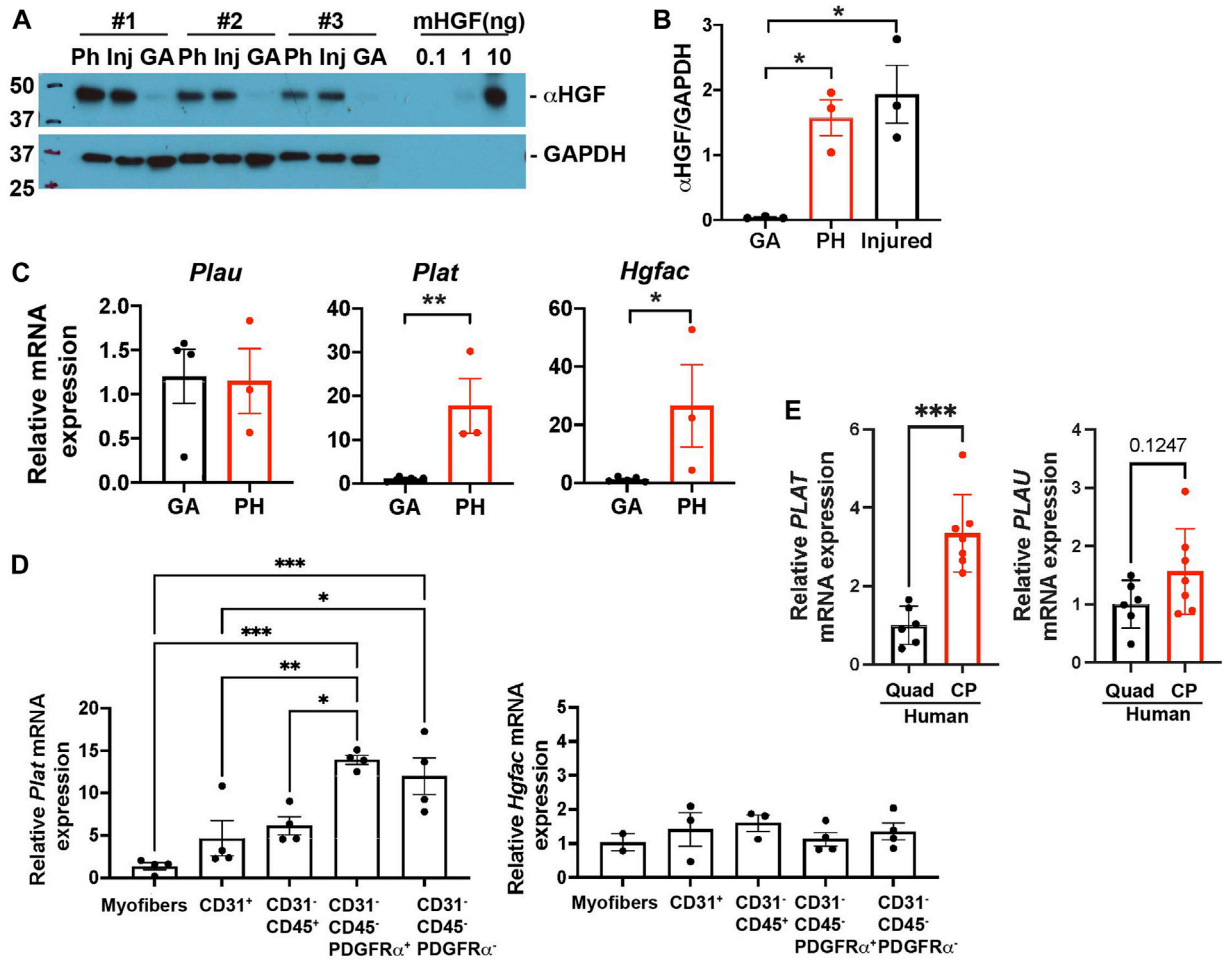


**FIGURE 5 |** Pharyngeal muscles contain an increased level of active HGF. **(A)** Western blot analyses of the active form of HGF (αHGF) protein, and GAPDH in gastrocnemius (GA), pharyngeal (PH), and 3-day $BaCl_2$-injured tibialis anterior (Inj) muscles. $n = 3$. **(B)** Normalized band intensity of the active form of HGF (αHGF) to GAPDH in gastrocnemius (GA) and pharyngeal (PH) muscles. 3-day $BaCl_2$-injured tibialis anterior muscles are included as a positive control. $n = 3$. **(C)** The relative mRNA expression level of urokinase-type plasminogen activator (*Plau*), tissue-type plasminogen activator (*Plat*), and HGF activator (*Hgfac*) in gastrocnemius (GA) and pharyngeal (PH) muscles obtained from 3 months old C57BL/6 mice. $n = 3$–4. **(D)** The relative mRNA expression level of tissue-type plasminogen activator (*Plat*) and HGF activator (*Hgfac*) in myofibers and sorted $CD31^+$, $CD31^-/CD45^+$, $CD31^-/CD45^-/PDGFR\alpha^+$, and $CD31^-/CD45^-/PDGFR\alpha^-$ cells obtained from muscles of 3 months old C57BL/6 mice. $n = 3$–4. Statistical significance was determined by 1-way ANOVA **(B, D)**, by student's t-test (C except *Hgfac*, **(E)**, or by Mann-Whitney test (*Hgfac* in C). For all graphs, the value represents mean ± SEM. Asterisks indicate statistical significance ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

promoting the unusual activities of pharyngeal and extraocular SCs.

## Pharyngeal Muscles Contain Increased Levels of Active Hepatocyte Growth Factor

HGF exists in a biologically inactive form in the extracellular matrix (ECM) of uninjured tissues (Gak et al., 1992). To actively mediate signaling, HGF is converted from an inactive precursor (pro-HGF) into a processed active form of HGF (active HGF, αHGF). To elucidate whether HGF is active in pharyngeal muscles, we used immunoblotting to measure active HGF by size as compared to mouse HGF standard proteins (**Figure 6A**, right side of blot). We detected activated HGF in pharyngeal and 3 day-injured TA muscles but not in gastrocnemius muscles (**Figures 5A,B**). Given that activation of HGF depends on cleavage by proteases, we measured multiple proteases in pharyngeal muscles. In injured muscles, urokinase plasminogen activators (PLAU) (Sisson et al., 2009) activate quiescent SCs and HGF activators (HGFAC) process HGF to promote the SC $G_{alert}$ state in distant uninjured muscles (Rodgers et al., 2017). The tissue-type plasminogen activator PLAT activates HGF but has not been studied in the context of injured muscles. However, elevated *Plat* transcript levels were detected in pharyngeal SCs compared to limb SCs in previously published microarray data (Randolph et al., 2015). To determine which proteases likely generate active HGF in pharyngeal muscles, we measured the levels of *Plau*, *Plat*, and *Hgfac* mRNAs. The levels of *Plat* and *Hgfac* mRNAs were increased in whole pharyngeal muscles relative to gastrocnemius muscles (**Figure 5C**), indicating that HGF in pharyngeal muscles could be activated by PLAT or HGFAC rather

than PLAU. In addition, *Plat* mRNA levels were increased in isolated FAPs ($CD31^-/CD45^-/PDGFR\alpha^+$), but *Hgfac* mRNA levels were broadly detected among the different cell types (**Figure 5D**). As we observed in mouse pharyngeal FAPs, human CP muscle also contain higher *PLAT* transcript levels compared to limb muscle but *PLAU* transcript levels are similar between human CP and limb muscles (**Figure 5E**). Taken together, these data suggest that pharyngeal FAPs are a major cell source of the HGF-activating enzyme PLAT and of HGF, which likely is responsible for the increased activation of pharyngeal SCs *in vivo*.

## Fibroadipogenic Progenitors are Responsible for Pharyngeal Satellite Cell Proliferation Through Hepatocyte Growth Factor

Our data revealed that elevated numbers of FAPs are responsible for the high levels of HGF detected in pharyngeal muscles (**Figure 3B**). To investigate if pharyngeal FAPs indeed secrete more HGF protein compared to gastrocnemius FAPs, we measured *Hgf* mRNA levels and secreted HGF protein levels. We found that *Hgf* mRNA levels are trended towards higher ($p = 0.11$) in FAPs derived from pharyngeal muscle compared to FAPs derived from gastrocnemius muscle (**Figure 6A**). Conditioned medium from pharyngeal FAPs (FAPs CM) also contained 3-fold higher levels of HGF protein compared to conditioned medium from gastrocnemius FAPs as measured by ELISA (**Figure 6B**). To confirm the contribution of FAPs to HGF-mediated pharyngeal SC proliferation, we generated FAPs-ablated mice using PDGFRα $Cre^{ERT}$-DTA mouse, which allows for the specific and conditional depletion of FAPs following tamoxifen-induced expression of diphtheria toxin (**Figure 6C**). Following tamoxifen treatment, pharyngeal muscles from PDGFRα $Cre^{ERT}$-DTA mice contained −80% less *Pdgfra* transcript in the pharyngeal muscle, confirming the effective FAPs ablation (**Figure 6D** left). Levels of *Hgf* mRNA (**Figure 6D** right) and HGF protein (**Figures 6E,F**) were significantly lower in FAPs-ablated pharyngeal muscles, suggesting that FAPs provide the majority of HGF in pharyngeal muscles. Furthermore, the percentage of $BrdU^+$ proliferating SCs was significantly lower in FAPs-ablated pharyngeal muscles compared to the control group (**Figure 6G**), indicating that FAPs promote the proliferation of uninjured pharyngeal SCs. However, basal proliferation of gastrocnemius SCs was not reduced by FAPs-ablation (**Figure 6H**).

## Fibroadipogenic Progenitors are Critical for Pharyngeal Muscle Function and Homeostasis

We next measured how FAPs depletion affected pharyngeal muscle function and homeostasis. In addition to their function in HGF secretion and SC proliferation, FAPs have been shown to play a key role in muscular regeneration *via* follistatin (Iezzi et al., 2004; Mozzetta et al., 2013) and muscle maintenance *via* BMP3B (Wosczyna et al., 2019; Uezumi et al., 2021). We performed pharyngeal muscle functional assays on 6-month old tamoxifen-treated PDGFRα $Cre^{ERT}$-DTA (FAPs-ablated) mice at 1, 2, and 3 months after FAPs depletion (**Figure 7A**). Depletion of $PDGFR\alpha^+$ cells was confirmed by immunofluorescence staining using PDGFRα antibodies on pharyngeal muscle sections (**Figures 7B,C**). FAPs-ablated mice showed significantly reduced lick rates at both 2 and 3 months after ablation (**Figure 7D**), suggesting they have reduced liquid swallowing ability. Along with a reduced lick rate, food consumption was significantly decreased in FAPs-ablated mice at all time points measured (**Figure 7E**). Water consumption was increased after 2 months of FAPs ablation (**Figure 7F**), which may represent compensatory consumption due to the reduced food intake. As a consequence, FAP-ablated mice showed decreased body weight (**Figure 7G**). Consistent with the functional deficits, the pharyngeal muscle cross-sectional area was significantly decreased in FAPs ablated mice (**Figures 7H,I**). In addition to loss of the established BMP3B-mediated mechanism of FAPs-regulated muscle homeostasis and neuromuscular integrity (Uezumi et al., 2021), the reduced food intake in FAP-ablated mice likely contributed to further limb muscle loss after FAPs depletion (**Figures 7J,K**) (Wosczyna et al., 2019; Uezumi et al., 2021). Although we detected reduced SCs proliferation after FAPs ablation (**Figures 6G,H**), loss of SC activity was not responsible for defective pharyngeal muscle function since SCs ablation had minimal effect on cricopharyngeal muscle size and swallowing function (**Supplementary Figure S6C–G**). Taken together, these results show that depletion of FAPs impairs pharyngeal muscle function and homeostasis thus demonstrating the importance of FAPs in pharyngeal muscles along with the detrimental role of FAPs in fibrotic pharyngeal muscles of OPMD patients (Bensalah et al., 2022).

# DISCUSSION

Although craniofacial muscles, including pharyngeal muscles, differ from limb and trunk muscles in embryonic origin and core genetic programs (Tajbakhsh, 2009), most of what we know about SCs, FAPs, and other cell types comes from studies in limb muscles. Studies of SCs and FAPs in craniofacial muscle are difficult due to the small size of craniofacial muscles, the difficult dissection, and the lack of functional assays. We employed genetic mouse models to reveal the distinct characteristics of pharyngeal SCs and to demonstrate the role of FAPs for pharyngeal SC proliferation and pharyngeal muscle homeostasis. To explain the highly proliferative and differentiative properties of pharyngeal SCs *in vivo*, we characterized pharyngeal SCs and investigated the role of the pharyngeal muscle environment for SC proliferation. Our study provides new evidence to explain how niche factors, such as HGF, and neighboring cells including FAPs and resident macrophages, govern the unique state of craniofacial SCs (**Figure 8**).

## Hepatocyte Growth Factor Contributes to the Activation of Pharyngeal Satellite Cells

Neighboring cells and SC contribute to the muscle microenvironment *via* soluble factors or direct cell-to-cell contact. HGF is one such auto/paracrine factor involved in SC activation in response to muscle injury, overuse, or mechanical stretches (Tatsumi et al., 1998; Miller et al., 2000; Sheehan et al.,

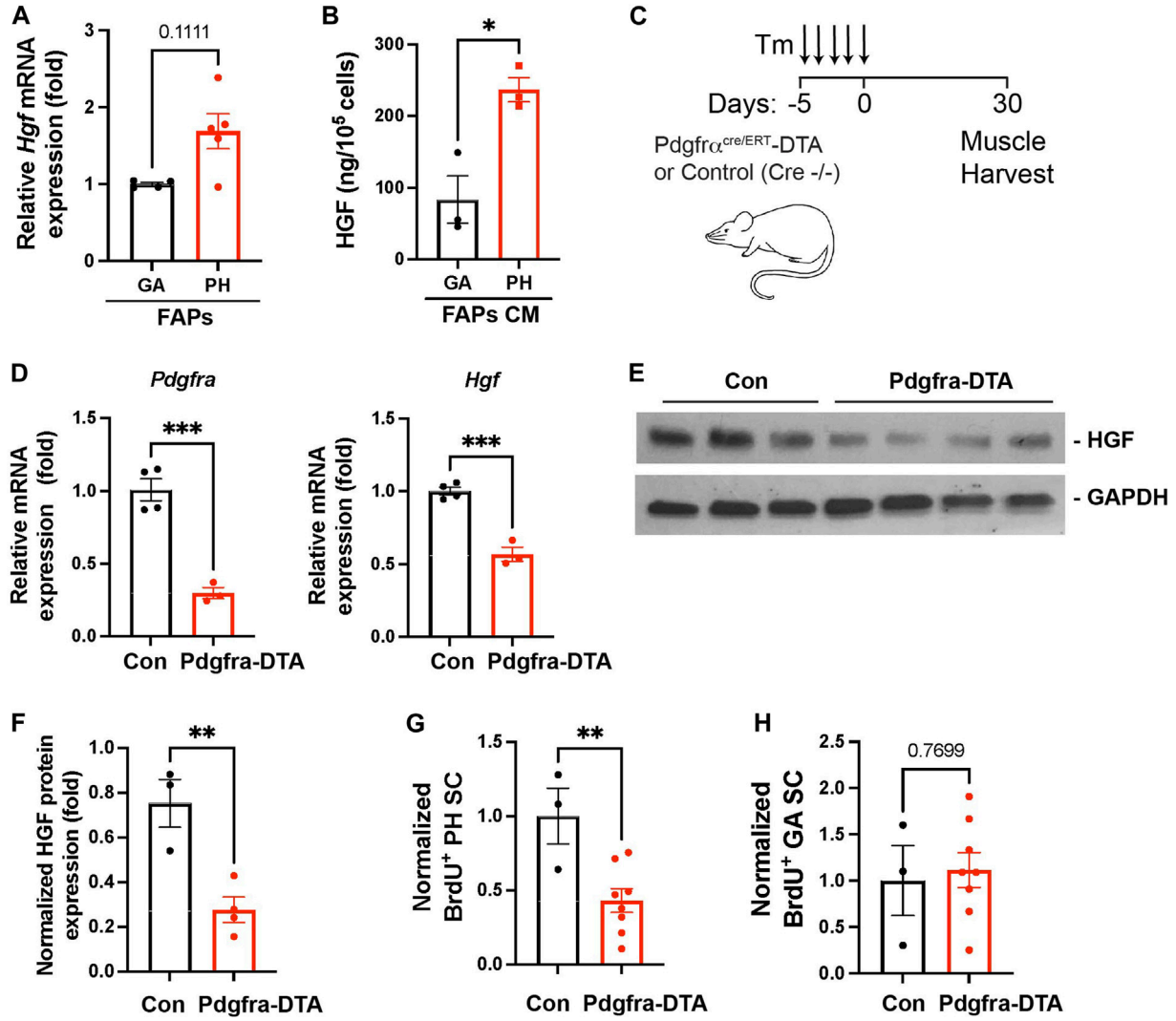


**FIGURE 6 |** Depleted FAPs reduce HGF expression and the proliferating SC population in pharyngeal muscles. **(A)** Relative mRNA expression level hepatocyte growth factor (*Hgf*) in freshly sorted CD31$^-$/CD45$^-$/PDGFRα$^+$ fibroadipogenic progenitors (FAPs) from gastrocnemius (GA) and pharyngeal (PH) muscles by MACS. $n$ = 4–5. **(B)** Concentration of HGF in cultured medium (CM) of the gastrocnemius (GA) and pharyngeal (PH) muscles-derived fibroadipogenic progenitors (FAPs). HGF was measured by ELISA and the amount of HGF was normalized with cell number. $n$ = 3. **(C)** Scheme of strategy for FAPs ablation using Pdgfrα *Cre$^{ERT}$*-DTA mouse. Tamoxifen (Tm). We used control (*Pdgfrα Cre$^{ERT-/-}$ -DTA*) and Pdgfra-DTA (*Pdgfrα Cre$^{ERT+/-}$ -DTA*) mice after 30 days of Tm injection. **(D)** The relative mRNA expression level of *Pdgfra*, and *Hgf* in pharyngeal (PH) muscles obtained from *Pdgfrα Cre$^{ERT}$ -DTA* mouse compared to control mice. $n$ = 3–4. **(E)** Western blot analyses of the active form of HGF (αHGF) protein, and GAPDH in pharyngeal muscles of control and Pdgfra-DTA mice. $n$ = 3–4. **(F)** Normalized band intensity of the active form of HGF (αHGF) to GAPDH in pharyngeal (PH) muscles. $n$ = 3–4. **(G, H)** Normalized percentage of BrdU$^+$ pharyngeal or gastrocnemius satellite cells (CD31$^-$/CD45$^-$/Sca1$^-$/Intergrin α7$^+$) isolated from Pdgfrα *Cre$^{ERT}$* -DTA mice compared to control mice. $n$ = 3 or 8. Statistical significance was determined by Mann-Whitney test **(A)** or by Student's t-test **(B, D) (F–H)**. The value represents mean ± SEM. Asterisks indicate statistical significance (*$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001).

2000; Tatsumi, 2010). HGF is secreted into the extracellular matrix of uninjured muscles as pro-HGF and is activated by proteolysis mediated by urokinase-type plasminogen activator (PLAU) (Sisson et al., 2009) or by circulating HGF activators originating from a remote muscle injury (Rodgers et al., 2017). Cleaved HGF, in turn, activates SCs (Stoker et al., 1987; Bernet-Camard et al., 1996; Sisson et al., 2009). In pharyngeal muscles, we detected high levels of the transcripts encoding both HGF activator (*Hgfac*) and tissue-type plasminogen activator (*Plat*), which is similar (identity 32.8% and similarity 43%) to the urokinase plasminogen activator that cleaves pro-HGF (Mars et al., 1993). However, the transcript encoding urokinase plasminogen activator (*Plau*) was not increased in pharyngeal muscles. This result indicates that uninjured pharyngeal muscles use a mechanism of SC activation, that is, distinct from that of the limb. Our previous microarray data comparing pharyngeal SCs with limb SCs (Randolph et al., 2015) revealed increased levels of the *Plat* transcript, suggesting that pharyngeal SCs also contribute to pro-HGF processing. Interestingly, extraocular muscle SCs, which also proliferate and differentiate without injury, also

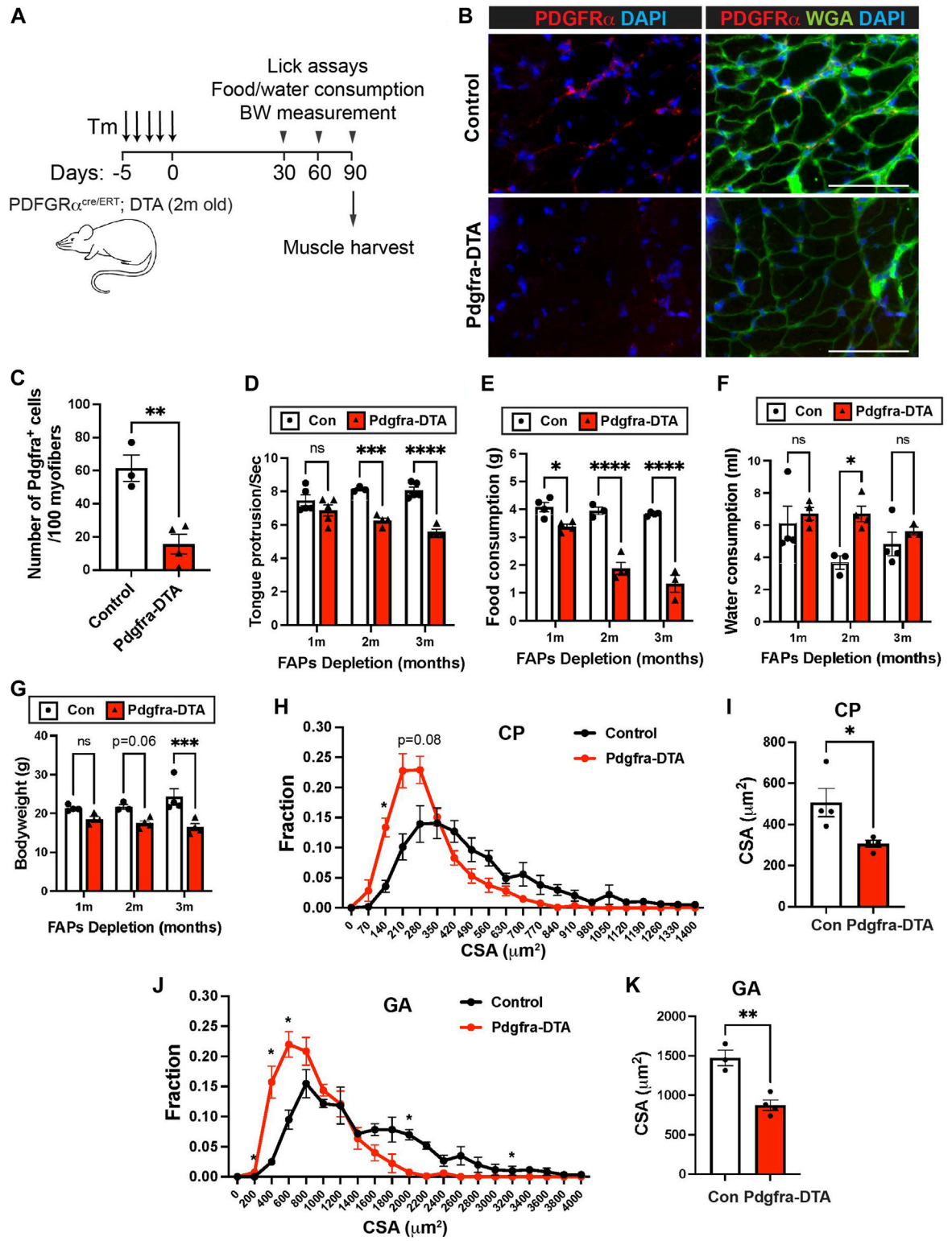


**FIGURE 7 |** FAPs are critical for pharyngeal muscle function and maintenance. **(A)** Scheme of strategy for satellite cell ablation using Pdgfra *Cre*$^{ERT+/-}$-DTA (Pdgfra-DTA, $n$ = 3–4) and Pdgfra *Cre* $^{ERT-/-}$-DTA (sex- and age-matched control, $n$ = 3–4). Both groups were injected with Tamoxifen (Tm). Lick assay was performed at 1, 2, and 3 months post tamoxifen injection using the same cohort. Mice were sacrificed at 3 months of FAPs ablation for muscle harvest. **(B)** Representative images of PDGFRα$^+$ cells in pharyngeal (PH) muscles of control and Pdgfra-DTA mice after 3 months of tamoxifen injection. Merged images show immunostaining with anti-PDGFRα (Red) and anti-laminin (green) antibodies and DAPI (blue). Scale bars = 70 μm. **(C)** The quantified number of PDGFRα$^+$ cells per 100 myofibers. $n$ = 4. **(D)** The

(*Continued*)

**FIGURE 7 |** number of tongue protrusions per second was counted when a mouse lick the water sipper using video analysis. **(E, F)** Each mouse was housed in a single cage to measure daily food (gram) and water (ml) consumption. **(G)** Bodyweight was measured at each month. **(H, J)** The cross-sectional area of CP or GA muscle fibers was measured between FAPs-ablated mice and control mice. **(I, K)** The average cross-sectional area of CP or GA muscle fibers. $n$ = 3–4. Statistical significance was determined by Student's t-test **(C, I, K)**, by multiple unpaired t-test for each size **(H, J)** or by 2-way ANOVA and Sidak's multiple comparison test **(D–G)**. The value represents mean ± SEM. Asterisks indicate statistical significance (*$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001).

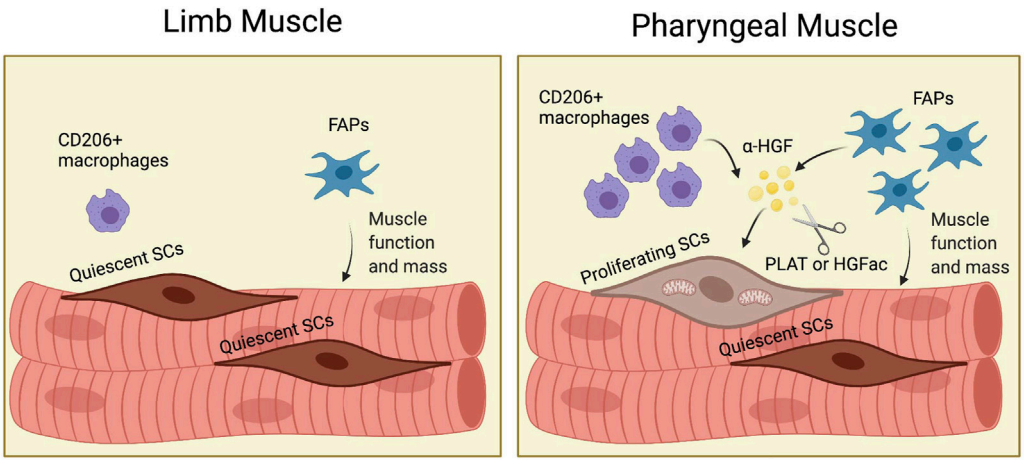


**FIGURE 8 |** (Graphic abstract). FAPs induce proliferation of pharyngeal satellite cells by HGF and maintain pharyngeal muscle mass and function. In pharyngeal muscles, the increased number of CD206$^+$ macrophages and fibroadipogenic progenitors (FAPs) secrete HGF. HGF is converted to the active form (α-HGF) by tissue-type plasminogen activator (PLAT) or HGF activator (HGFac). Therefore, FAPs mediate proliferation of SCs in pharyngeal muscles *via* HGF. In addition, FAPs maintain pharyngeal muscle mass and function. Created with BioRender.com.

contain high levels of *Plat* mRNA relative to limb SCs (Pacheco-Pinedo et al., 2009). In addition, extraocular muscles express high levels of *Hgf* mRNA. Thus, HGF is likely an important signal that modulates both pharyngeal and extraocular SC activity. Additional studies are needed to better define the unique mechanisms of pro-HGF processing and HGF-mediated craniofacial SC activation.

## Fibroadipogenic Progenitors Release Hepatocyte Growth Factor in Pharyngeal Muscles

Muscle is a heterogeneous tissue that contains myofibers, SCs, blood vessels, peripheral nerves, mesenchymal cells, and resident macrophages (Bentzinger et al., 2013; Tedesco et al., 2017). To identify the major source of HGF, we sorted myofibers, endothelial cells, immune cells, and others using MACS. We also ruled out the autocrine secretion of HGF from pharyngeal SCs using SC-ablated mice, which is consistent with the previous microarray analysis showing that pharyngeal SCs contain comparable *Hgf* mRNA levels to limb SCs (Randolph et al., 2015). We found the highest levels of *Hgf* mRNA in the CD31$^-$/CD45$^-$/PDGFRα$^+$ fraction isolated by MACS. Considering that FAPs are the main population of the CD31$^-$/CD45$^-$/PDGFRα$^+$ fraction, we focused on FAPs as the likely source of HGF in pharyngeal muscle niche. Pharyngeal muscles contain a high number of FAPs, which may be a result of pro-proliferative HGF autocrine signaling (Rodgers et al., 2017). Importantly, we discovered that FAPs are the major source of elevated HGF detected in pharyngeal muscles and are responsible for the active proliferation of pharyngeal SCs in FAPs ablation experiments. In addition to results obtained from mice, we confirmed an increased *HGF*, *PDGFRa*, and *PLAT* transcript levels and CD90$^+$ FAPs in human CP muscles. The increased *HGF* levels in CP is consistent with a previous report examining several craniofacial muscles, including CP muscles from 14 cadavers (Rhee et al., 2016). These data indicate the translational potential of our data generated in mice. Like SCs, FAPs are more proliferative in pharyngeal muscles compared to limb muscles in mouse and human (Bensalah et al., 2022), which could be related to the high level of extracellular matrix (ECM) found in pharyngeal muscles compared to limb muscles in healthy humans (Gidaro et al., 2013; Bensalah et al., 2022). Future studies are needed to probe how FAPs are activated and differentiated in pharyngeal muscles, whether this activation involves interaction with CD206$^+$ macrophages or HGF autocrine effects, and whether this regulation is disturbed in pharyngeal muscle-specific pathologies with excessive ECM like OPMD (Gidaro et al., 2013; Bensalah et al., 2022).

In regenerating muscles, the majority of HGF is secreted by macrophages (Sisson et al., 2009) that infiltrate the skeletal muscle niche after injury (Pillon et al., 2013). Indeed, a large number of CD206$^+$ macrophages in pharyngeal muscles are detected and contribute to the high level of *Hgf* transcript, but these are likely tissue-resident rather than infiltrating

macrophages because pharyngeal muscles show no signs of injury (Randolph et al., 2015) and have low levels of infiltrating Cx3cr1+ macrophages. Resident macrophages in skeletal muscles express CD206, as well as CD45, CD11b, F4/80, and CD64, and exhibit distinct transcriptional profiles when depending on whether they were isolated from limb versus diaphragm muscles (Wang et al., 2020). It is unclear why CP muscles contain high numbers of FAPs and CD206$^{+}$ macrophages, but the pharyngeal muscle niche may play a role in the recruitment of both cells. We did not detect any signs of injury, such as clusters of immune cells, in pharyngeal muscles. However, pharyngeal muscle contains relatively high levels of centrally nucleated fibers, which we previously interpreted as a sign of continuous satellite cell fusion. Alternatively, centrally nucleated fibers may be a result of muscle turnover in pharyngeal muscles that promotes FAPs and macrophage proliferation and proliferates satellite cells. Although we did not observe embryonic myosin heavy chain$^{+}$ myofibers, neonatal myosin heavy chain protein was detected in pharyngeal muscles using immunoblotting (Randolph et al., 2015). Given the known role of resident macrophages in skeletal and cardiac muscles (Brigitte et al., 2010; Bajpai et al., 2019; Theret et al., 2021), we speculate that CD206$^{+}$ macrophages may regulate pharyngeal muscle homeostasis and its responses to inflammation.

## Role of Fibroadipogenic Progenitors in Craniofacial Muscles

Similar to the distinctive embryonic origins of craniofacial muscles and SCs, craniofacial FAPs are derived from cranial neural crests, while FAPs in limb/trunk muscles arise from lateral plate mesoderm-derived somites (Sefton and Kardon, 2019). Neural crest-derived muscle connective tissues, precursors of FAPs, are required for craniofacial muscle development and morphogenesis (Noden and Trainor, 2005; Rinon et al., 2007). Although less well studied, FAPs were thought to have a profound role in craniofacial muscle homeostasis and regeneration (Cheng et al., 2021). Interestingly, HGF is one of the critical factors that induce migration of muscle progenitor cells that express cMET as an HGF receptor for muscle development (Bladt et al., 1995; Dietrich et al., 1999). Given the role of muscle connective tissues during development, they influence the migration of muscle progenitor cells *via* HGF. Although we identified that FAPs are responsible for high levels of HGF and unusual SC proliferation in pharyngeal muscles, we still do not understand the functional importance of the proliferating SCs in adult pharyngeal muscles. We speculate that a subset of proliferating SCs could be influenced by HGF and other factors secreted by local FAPs. While we can not exclude intrinsic factors of SC for pharyngeal muscle homeostasis, we found satellite cell-ablated mice present normal swallowing function and pharyngeal muscle size, the role of satellite cells for pharyngeal muscles may not be critical at least in physiologic conditions. However, their distinct behaviors, such as proliferation without injury and continuous fusion to pharyngeal and extraocular muscles, should be analyzed in craniofacial muscle-specific diseases, such as oculopharyngeal muscular dystrophy (OPMD). In contrast to the dispensable role of SCs for pharyngeal muscle function, we found FAPs to be critical for pharyngeal muscle function and maintenance. Impaired pharyngeal muscle function may explain rapid loss of body weight and muscle mass following FAPs depletion in our study and previous study (Wosczyna et al., 2019; Uezumi et al., 2021). However, heterogeneous origins and a lack of specific markers are challenges in investigating craniofacial FAPs. In this study, we used a PDGFRα $Cre^{ERT}$-DTA mouse model to investigate the role of FAPs in the basal proliferation of SC in pharyngeal muscles. While pharyngeal SCs showed less proliferation with FAPs depletion in PDGFRα $Cre^{ERT}$-DTA mouse, we cannot exclude the influence of removed stromal cells in other tissues of this mouse model. Localized FAPs-depletion (Wosczyna et al., 2019) would not be feasible due to the deep location and thin structure of pharyngeal muscles that would make impossible to access with locally-injected AAV.

In conclusion, this study is the first report to identify niche/environmental factors associated with the highly proliferative features of pharyngeal SCs. While it is not clear whether the unique embryonic origins of pharyngeal muscles lead to the differences in proliferative and myogenic properties of SCs, this study demonstrates that the pharyngeal muscle niche, such as FAPs and HGF from FAPs, is capable of proliferating pharyngeal SCs without injury. Although the role of highly active SCs in pharyngeal muscle function is still ambiguous, we propose that unique properties of pharyngeal SCs and FAPs would provide useful information to understand pharyngeal muscle-specific pathologies, such as oculopharyngeal muscular dystrophy and dysphagia.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the French Ministry of Research. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Emory University's Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

EK designed the study, performed experiments, image analysis, analyzed data, prepared figures, and wrote the manuscript. FW performed experiments, analyzed data and wrote the manuscript. DL performed experiments, image analysis, and analyzed data.

CZ performed experiments, image analysis, and analyzed data. YZ performed experiments and image analysis. JA prepared samples and illustrated a pharynx image for **Figure 1**. LM performed experiments and analyzed data. CT provided human samples, and revised the manuscript. KV wrote and revised the manuscript. HC designed the study, performed experiments and image analysis, analyzed data, prepared figures, and wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.875209/full#supplementary-material

## REFERENCES

Allen, R. E., Sheehan, S. M., Taylor, R. G., Kendall, T. L., and Rice, G. M. (1995). Hepatocyte Growth Factor Activates Quiescent Skeletal Muscle Satellite Cells *In Vitro*. *J. Cell. Physiol.* 165 (2), 307–312. doi:10.1002/jcp.1041650211

Arenaz Búa, B., Olsson, R., Westin, U., Rydell, R., and Ekberg, O. (2015). Treatment of Cricopharyngeal Dysfunction: a Comparative Pilot Study. *BMC Res. Notes* 8 (1), 301. doi:10.1186/s13104-015-1266-x

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., et al. (2007). Inflammatory Monocytes Recruited after Skeletal Muscle Injury Switch into Antiinflammatory Macrophages to Support Myogenesis. *J. Exp. Med.* 204 (5), 1057–1069. doi:10.1084/jem.20070075

Baghdadi, M. B., Castel, D., Machado, L., Fukada, S.-i., Birk, D. E., Relaix, F., et al. (2018). Reciprocal Signalling by Notch-Collagen V-CALCR Retains Muscle Stem Cells in Their Niche. *Nature* 557 (7707), 714–718. doi:10.1038/s41586-018-0144-9

Bajpai, G., Bredemeyer, A., Li, W., Zaitsev, K., Koenig, A. L., Lokshina, I., et al. (2019). Tissue Resident CCR2− and CCR2+ Cardiac Macrophages Differentially Orchestrate Monocyte Recruitment and Fate Specification Following Myocardial Injury. *Circ. Res.* 124 (2), 263–278. doi:10.1161/circresaha.118.314028

Bensalah, M., Muraine, L., Boulinguiez, A., Giordani, L., Albert, V., Ythier, V., et al. (2022). A Negative Feedback Loop between Fibroadipogenic Progenitors and Muscle Fibres Involving Endothelin Promotes Human Muscle Fibrosis. *J. Cachexia Sarcopenia Muscle* 1, 1. doi:10.1002/jcsm.12974

Bentzinger, C. F., Wang, Y. X., Dumont, N. A., and Rudnicki, M. A. (2013). Cellular Dynamics in the Muscle Satellite Cell Niche. *EMBO Rep.* 14 (12), 1062–1072. doi:10.1038/embor.2013.182

Bernet-Camard, M. F., Coconnier, M. H., Hudault, S., and Servin, A. L. (1996). Differential Expression of Complement Proteins and Regulatory Decay Accelerating Factor in Relation to Differentiation of Cultured Human Colon Adenocarcinoma Cell Lines. *Gut* 38 (2), 248–253. doi:10.1136/gut.38.2.248

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995). Essential Role for the C-Met Receptor in the Migration of Myogenic Precursor Cells into the Limb Bud. *Nature* 376 (6543), 768–771. doi:10.1038/376768a0

Bosnakovski, D., Xu, Z., Li, W., Thet, S., Cleaver, O., Perlingeiro, R. C. R., et al. (2008). Prospective Isolation of Skeletal Muscle Stem Cells with a Pax7 Reporter. *Stem Cells* 26 (12), 3194–3204. doi:10.1634/stemcells.2007-1017

Brigitte, M., Schilte, C., Plonquet, A., Baba-Amer, Y., Henri, A., Charlier, C., et al. (2010). Muscle Resident Macrophages Control the Immune Cell Reaction in a Mouse Model of Notexin-Induced Myoinjury. *Arthritis Rheum.* 62 (1), 268–279. doi:10.1002/art.27183

Cheng, X., Shi, B., and Li, J. (2021). Distinct Embryonic Origin and Injury Response of Resident Stem Cells in Craniofacial Muscles. *Front. Physiology* 12, 690248. doi:10.3389/fphys.2021.690248

Choi, W., Lee, J., Lee, J., Lee, S. H., and Kim, S. (2019). Hepatocyte Growth Factor Regulates Macrophage Transition to the M2 Phenotype and Promotes Murine Skeletal Muscle Regeneration. *Front. Physiol.* 10, 914. doi:10.3389/fphys.2019.00914

Choo, H.-J., Cutler, A., Rother, F., Bader, M., and Pavlath, G. K. (2016). Karyopherin Alpha 1 Regulates Satellite Cell Proliferation and Survival by Modulating Nuclear Import. *Stem Cells* 34 (11), 2784–2797. doi:10.1002/stem.2467

Dick, S. A., Wong, A., Hamidzada, H., Nejat, S., Nechanitzky, R., Vohra, S., et al. (2022). Three Tissue Resident Macrophage Subsets Coexist across Organs with Conserved Origins and Life Cycles. *Sci. Immunol.* 7 (67), eabf7777. doi:10.1126/sciimmunol.abf7777

Dietrich, S., Abou-Rebyeh, F., Brohmann, H., Bladt, F., Sonnenberg-Riethmacher, E., Yamaai, T., et al. (1999). The Role of SF/HGF and C-Met in the Development of Skeletal Muscle. *Development* 126 (8), 1621–1629. doi:10.1242/dev.126.8.1621

Evano, B., and Tajbakhsh, S. (2018). Skeletal Muscle Stem Cells in Comfort and Stress. *NPJ Regen. Med.* 3 (1), 24–13. doi:10.1038/s41536-018-0062-3

Formicola, L., Marazzi, G., and Sassoon, D. A. (2014). The Extraocular Muscle Stem Cell Niche Is Resistant to Ageing and Disease. *Front. Aging Neurosci.* 6, 328. doi:10.3389/fnagi.2014.00328

Fry, C. S., Lee, J. D., Mula, J., Kirby, T. J., Jackson, J. R., Liu, F., et al. (2015). Inducible Depletion of Satellite Cells in Adult, Sedentary Mice Impairs Muscle Regenerative Capacity without Affecting Sarcopenia. *Nat. Med.* 21 (1), 76–80. doi:10.1038/nm.3710

Gak, E., Taylor, W. G., Chan, A. M.-L., and Rubin, J. S. (1992). Processing of Hepatocyte Growth Factor to the Heterodimeric Form Is Required for Biological Activity. *FEBS Lett.* 311 (1), 17–21. doi:10.1016/0014-5793(92)81356-q

Gharaibeh, B., Lu, A., Tebbets, J., Zheng, B., Feduska, J., Crisan, M., et al. (2008). Isolation of a Slowly Adhering Cell Fraction Containing Stem Cells from Murine Skeletal Muscle by the Preplate Technique. *Nat. Protoc.* 3 (9), 1501–1509. doi:10.1038/nprot.2008.142

Gidaro, T., Negroni, E., Perié, S., Mirabella, M., Lainé, J., St Guily, J. L., et al. (2013). Atrophy, Fibrosis, and Increased PAX7-Positive Cells in Pharyngeal Muscles of Oculopharyngeal Muscular Dystrophy Patients. *J. Neuropathol. Exp. Neurol.* 72 (3), 234–243. doi:10.1097/nen.0b013e3182854c07

Girardi, F., Taleb, A., Ebrahimi, M., Datye, A., Gamage, D. G., Peccate, C., et al. (2021). TGFβ Signaling Curbs Cell Fusion and Muscle Regeneration. *Nat. Commun.* 12 (1). doi:10.1038/s41467-020-20289-8

Gómez-Torres, A., Abrante Jiménez, A., Rivas Infante, E., Menoyo Bueno, A., Tirado Zamora, I., and Esteban Ortega, F. (2012). Cricopharyngeal Myotomy in the Treatment of Oculopharyngeal Muscular Dystrophy. *Acta Otorrinolaringol. Engl. Ed.* 63 (6), 465–469. doi:10.1016/j.otoeng.2012.11.009

Harel, I., Nathan, E., Tirosh-Finkel, L., Zigdon, H., Guimarães-Camboa, N., Evans, S. M., et al. (2009). Distinct Origins and Genetic Programs of Head Muscle Satellite Cells. *Dev. cell* 16 (6), 822–832. doi:10.1016/j.devcel.2009.05.007

Helmbacher, F., and Stricker, S. (2020). Tissue Cross Talks Governing Limb Muscle Development and Regeneration. *Seminars Cell & Dev. Biol.* 104, 14–30. doi:10.1016/j.semcdb.2020.05.005

Heredia, J. E., Mukundan, L., Chen, F. M., Mueller, A. A., Deo, R. C., Locksley, R. M., et al. (2013). Type 2 Innate Signals Stimulate Fibro/adipogenic Progenitors to Facilitate Muscle Regeneration. *Cell* 153 (2), 376–388. doi:10.1016/j.cell.2013.02.053

Iezzi, S., Di Padova, M., Serra, C., Caretti, G., Simone, C., Maklan, E., et al. (2004). Deacetylase Inhibitors Increase Muscle Cell Size by Promoting Myoblast Recruitment and Fusion through Induction of Follistatin. *Dev. Cell* 6 (5), 673–684. doi:10.1016/s1534-5807(04)00107-8

Jansen, K. M., and Pavlath, G. K. (2006). Mannose Receptor Regulates Myoblast Motility and Muscle Growth. *J. cell Biol.* 174 (3), 403–413. doi:10.1083/jcb.200601102

Joe, A. W. B., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., et al. (2010). Muscle Injury Activates Resident Fibro/adipogenic Progenitors that Facilitate Myogenesis. *Nat. Cell Biol.* 12 (2), 153–163. doi:10.1038/ncb2015

Keefe, A. C., Lawson, J. A., Flygare, S. D., Fox, Z. D., Colasanto, M. P., Mathew, S. J., et al. (2015). Muscle Stem Cells Contribute to Myofibres in Sedentary Adult Mice. *Nat. Commun.* 6 (1). doi:10.1038/ncomms8087

Kelly, R. G., Jerome-Majewska, L. A., and Papaioannou, V. E. (2004). The del22q11.2 candidate gene Tbx1 regulates branchiomeric myogenesis. *Hum. Mol. Genet.* 13 (22), 2829–2840. doi:10.1093/hmg/ddh304

Khurana, T. S., Prendergast, R. A., Alameddine, H. S., Tomé, F. M., Fardeau, M., Arahata, K., et al. (1995). Absence of Extraocular Muscle Pathology in Duchenne's Muscular Dystrophy: Role for Calcium Homeostasis in Extraocular Muscle Sparing. *J. Exp. Med.* 182 (2), 467–475. doi:10.1084/jem.182.2.467

Lepper, C., Conway, S. J., and Fan, C.-M. (2009). Adult Satellite Cells and Embryonic Muscle Progenitors Have Distinct Genetic Requirements. *Nature* 460 (7255), 627–631. doi:10.1038/nature08209

Lepper, C., Partridge, T. A., and Fan, C.-M. (2011). An Absolute Requirement for Pax7-Positive Satellite Cells in Acute Injury-Induced Skeletal Muscle Regeneration. *Development* 138 (17), 3639–3646. doi:10.1242/dev.067595

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *methods* 25 (4), 402–408. doi:10.1006/meth.2001.1262

Mars, W. M., Zarnegar, R., and Michalopoulos, G. K. (1993). Activation of Hepatocyte Growth Factor by the Plasminogen Activators uPA and tPA. *Am. J. Pathol.* 143 (3), 949–958.

Mauro, A. (1961). Satellite Cell of Skeletal Muscle Fibers. *J. biophysical Biochem. Cytol.* 9 (2), 493–495. doi:10.1083/jcb.9.2.493

McLoon, L. K., and Wirtschafter, J. (2002). Activated Satellite Cells Are Present in Uninjured Extraocular Muscles of Mature Mice. *Trans. Am. Ophthalmol. Soc.* 100, 119–124.

Miller, K. J., Thaloor, D., Matteson, S., and Pavlath, G. K. (2000). Hepatocyte Growth Factor Affects Satellite Cell Activation and Differentiation in Regenerating Skeletal Muscle. *Am. J. Physiology-Cell Physiology* 278 (1), C174–C181. doi:10.1152/ajpcell.2000.278.1.c174

Montarras, D., Morgan, J., Collins, C., Relaix, F., Zaffran, S., Cumano, A., et al. (2005). Direct Isolation of Satellite Cells for Skeletal Muscle Regeneration. *Science* 309 (5743), 2064–2067. doi:10.1126/science.1114758

Mootoosamy, R. C., and Dietrich, S. (2002). Distinct Regulatory Cascades for Head and Trunk Myogenesis. *Development* 129 (3), 573–583. doi:10.1242/dev.129.3.573

Motohashi, N., Uezumi, A., Yada, E., Fukada, S.-i., Fukushima, K., Imaizumi, K., et al. (2008). Muscle CD31(−) CD45(−) Side Population Cells Promote Muscle Regeneration by Stimulating Proliferation and Migration of Myoblasts. *Am. J. pathology* 173 (3), 781–791. doi:10.2353/ajpath.2008.070902

Mozzetta, C., Consalvi, S., Saccone, V., Tierney, M., Diamantini, A., Mitchell, K. J., et al. (2013). Fibroadipogenic Progenitors Mediate the Ability of HDAC Inhibitors to Promote Regeneration in Dystrophic Muscles of Young, but Not Old Mdx Mice. *EMBO Mol. Med.* 5 (4), 626–639. doi:10.1002/emmm.201202096

Murphy, M. M., Lawson, J. A., Mathew, S. J., Hutcheson, D. A., and Kardon, G. (2011). Satellite Cells, Connective Tissue Fibroblasts and Their Interactions Are Crucial for Muscle Regeneration. *Development* 138 (17), 3625–3637. doi:10.1242/dev.064162

Noden, D. M., and Francis-West, P. (2006). The Differentiation and Morphogenesis of Craniofacial Muscles. *Dev. Dyn.* 235 (5), 1194–1218. doi:10.1002/dvdy.20697

Noden, D. M., and Trainor, P. A. (2005). Relations and Interactions between Cranial Mesoderm and Neural Crest Populations. *J. Anat.* 207 (5), 575–601. doi:10.1111/j.1469-7580.2005.00473.x

Oustanina, S., Hause, G., and Braun, T. (2004). Pax7 Directs Postnatal Renewal and Propagation of Myogenic Satellite Cells but Not Their Specification. *Embo J.* 23 (16), 3430–3439. doi:10.1038/sj.emboj.7600346

Pacheco-Pinedo, E. C., Budak, M. T., Zeiger, U., Jørgensen, L. H., Bogdanovich, S., Schrøder, H. D., et al. (2009). Transcriptional and Functional Differences in Stem Cell Populations Isolated from Extraocular and Limb Muscles. *Physiol. Genomics* 37 (1), 35–42. doi:10.1152/physiolgenomics.00051.2008

Pillon, N. J., Bilan, P. J., Fink, L. N., and Klip, A. (2013). Cross-talk between Skeletal Muscle and Immune Cells: Muscle-Derived Mediators and Metabolic Implications. *Am. J. Physiology-Endocrinology Metabolism* 304 (5), E453–E465. doi:10.1152/ajpendo.00553.2012

Poot, M., and Pierce, R. H. (1999). Detection of Changes in Mitochondrial Function during Apoptosis by Simultaneous Staining with Multiple Fluorescent Dyes and Correlated Multiparameter Flow Cytometry. *Cytometry* 35 (4), 311–317. doi:10.1002/(sici)1097-0320(19990401)35:4<311::aid-cyto3>3.0.co;2-e

Randolph, M. E., Phillips, B. L., Choo, H.-J., Vest, K. E., Vera, Y., and Pavlath, G. K. (2015). Pharyngeal Satellite Cells Undergo Myogenesis under Basal Conditions and Are Required for Pharyngeal Muscle Maintenance. *Stem Cells* 33 (12), 3581–3595. doi:10.1002/stem.2098

Rhee, S., Yamamoto, M., Kitamura, K., Masaaki, K., Katori, Y., Murakami, G., et al. (2016). Macrophage Density in Pharyngeal and Laryngeal Muscles Greatly Exceeds that in Other Striated Muscles: an Immunohistochemical Study Using Elderly Human Cadavers. *Anat. Cell Biol.* 49 (3), 177–183. doi:10.5115/acb.2016.49.3.177

Rinon, A., Lazar, S., Marshall, H., Büchmann-Møller, S., Neufeld, A., Elhanany-Tamir, H., et al. (2007). Cranial Neural Crest Cells Regulate Head Muscle Patterning and Differentiation during Vertebrate Embryogenesis. *Development* 134 (17), 3065–3075. doi:10.1242/dev.002501

Rodgers, J. T., King, K. Y., Brett, J. O., Cromie, M. J., Charville, G. W., Maguire, K. K., et al. (2014). mTORC1 Controls the Adaptive Transition of Quiescent Stem Cells from G0 to GAlert. *Nature* 510 (7505), 393–396. doi:10.1038/nature13255

Rodgers, J. T., Schroeder, M. D., Ma, C., and Rando, T. A. (2017). HGFA Is an Injury-Regulated Systemic Factor that Induces the Transition of Stem Cells into G Alert. *Cell Rep.* 19 (3), 479–486. doi:10.1016/j.celrep.2017.03.066

Sambasivan, R., Gayraud-Morel, B., Dumas, G., Cimper, C., Paisant, S., Kelly, R. G., et al. (2009). Distinct Regulatory Cascades Govern Extraocular and Pharyngeal Arch Muscle Progenitor Cell Fates. *Dev. cell* 16 (6), 810–821. doi:10.1016/j.devcel.2009.05.008

Sambasivan, R., Kuratani, S., and Tajbakhsh, S. (2011). An Eye on the Head: the Development and Evolution of Craniofacial Muscles. *Development* 138 (12), 2401–2415. doi:10.1242/dev.040972

Sambasivan, R., Yao, R., Kissenpfennig, A., Van Wittenberghe, L., Paldi, A., Gayraud-Morel, B., et al. (2011). Pax7-expressing Satellite Cells Are Indispensable for Adult Skeletal Muscle Regeneration. *Development* 138 (17), 3647–3656. doi:10.1242/dev.067587

Sefton, E. M., and Kardon, G. (2019). Connecting Muscle Development, Birth Defects, and Evolution: An Essential Role for Muscle Connective Tissue. *Curr. Top. Dev. Biol.* 132, 137–176. doi:10.1016/bs.ctdb.2018.12.004

Sheehan, S. M., Tatsumi, R., Temm-Grove, C. J., and Allen, R. E. (2000). HGF Is an Autocrine Growth Factor for Skeletal Muscle Satellite Cells *In Vitro*. *Muscle Nerve* 23 (2), 239–245. doi:10.1002/(sici)1097-4598(200002)23:2<239::aid-mus15>3.0.co;2-u

Shi, X., and Garry, D. J. (2006). Muscle Stem Cells in Development, Regeneration, and Disease. *Genes Dev.* 20 (13), 1692–1708. doi:10.1101/gad.1419406

Sisson, T. H., Nguyen, M.-H., Yu, B., Novak, M. L., Simon, R. H., and Koh, T. J. (2009). Urokinase-type Plasminogen Activator Increases Hepatocyte Growth Factor Activity Required for Skeletal Muscle Regeneration. *Blood* 114 (24), 5052–5061. doi:10.1182/blood-2008-12-196212

Stoker, M., Gherardi, E., Perryman, M., and Gray, J. (1987). Scatter Factor Is a Fibroblast-Derived Modulator of Epithelial Cell Mobility. *Nature* 327 (6119), 239–242. doi:10.1038/327239a0

Stuelsatz, P., Shearer, A., Li, Y., Muir, L. A., Ieronimakis, N., Shen, Q. W., et al. (2015). Extraocular Muscle Satellite Cells Are High Performance Myo-Engines Retaining Efficient Regenerative Capacity in Dystrophin Deficiency. *Dev. Biol.* 397 (1), 31–44. doi:10.1016/j.ydbio.2014.08.035

Tajbakhsh, S. (2009). Skeletal Muscle Stem Cells in Developmental versus Regenerative Myogenesis. *J. Intern. Med.* 266 (4), 372–389. doi:10.1111/j.1365-2796.2009.02158.x

Tatsumi, R., Anderson, J. E., Nevoret, C. J., Halevy, O., and Allen, R. E. (1998). HGF/SF Is Present in Normal Adult Skeletal Muscle and Is Capable of Activating Satellite Cells. *Dev. Biol.* 194 (1), 114–128. doi:10.1006/dbio.1997.8803

Tatsumi, R. (2010). Mechano-biology of Skeletal Muscle Hypertrophy and Regeneration: Possible Mechanism of Stretch-Induced Activation of Resident Myogenic Stem Cells. *Animal Sci. J.* 81 (1), 11–20. doi:10.1111/j.1740-0929.2009.00712.x

Tedesco, F. S., Moyle, L. A., and Perdiguero, E. (2017). Muscle Interstitial Cells: a Brief Field Guide to Non-satellite Cell Populations in Skeletal Muscle. *Muscle Stem Cells* 1556, 129–147. doi:10.1007/978-1-4939-6771-1_7

Theret, M., Rossi, F., and Contreras, O. (2021). Evolving Roles of Muscle-Resident Fibro-Adipogenic Progenitors in Health, Regeneration, Neuromuscular Disorders, and Aging. *Front. Physiology* 12, 481. doi:10.3389/fphys.2021.673404

Uezumi, A., Ikemoto-Uezumi, M., Zhou, H., Kurosawa, T., Yoshimoto, Y., Nakatani, M., et al. (2021). Mesenchymal Bmp3b Expression Maintains Skeletal Muscle Integrity and Decreases in Age-Related Sarcopenia. *J. Clin. Invest* 131 (1). doi:10.1172/JCI139617

Victor, M., Hayes, R., and Adams, R. D. (1962). Oculopharyngeal Muscular Dystrophy. *N. Engl. J. Med.* 267 (25), 1267–1272. doi:10.1056/nejm196212202672501

Vishwakarma, A., Rouwkema, J., Jones, P. A., and Karp, J. M. (2017). The Need to Study, Mimic, and Target Stem Cell Niches. Biology and Engineering of Stem Cell Niches. *Biol. Eng. Stem Cell Nishes* 1, 3–13. doi:10.1016/b978-0-12-802734-9.00001-9

Walker, N., Kahamba, T., Woudberg, N., Goetsch, K., and Niesler, C. (2015). Dose-dependent Modulation of Myogenesis by HGF: Implications for C-Met Expression and Downstream Signalling Pathways. *Growth factors.* 33 (3), 229–241. doi:10.3109/08977194.2015.1058260

Wang, X., Sathe, A. A., Smith, G. R., Ruf-Zamojski, F., Nair, V., Lavine, K. J., et al. (2020). Heterogeneous Origins and Functions of Mouse Skeletal Muscle-Resident Macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 117 (34), 20729–20740. doi:10.1073/pnas.1915950117

Wosczyna, M. N., Konishi, C. T., Perez Carbajal, E. E., Wang, T. T., Walsh, R. A., Gan, Q., et al. (2019). Mesenchymal Stromal Cells Are Required for Regeneration and Homeostatic Maintenance of Skeletal Muscle. *Cell Rep.* 27 (7), 2029–2035. e5. doi:10.1016/j.celrep.2019.04.074

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# Differentiation Capacity of Human Urine-Derived Stem Cells to Retain Telomerase Activity

*Yingai Shi[1,2†], Guihua Liu[1,3†], Rongpei Wu[1,4], David L. Mack[1,5], Xiuzhi Susan Sun[1,6], Joshua Maxwell[1], Xuan Guan[1,7], Anthony Atala[1] and Yuanyuan Zhang[1]**

[1]Institute for Regenerative Medicine, Wake Forest University, Winston-Salem, NC, United States, [2]Key Laboratory of Pathobiology, Ministry of Education, College of Basic Medical Sciences, Jilin University, Jilin, China, [3]Reproductive Medical Center, Sixth Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China, [4]Department of Urology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China, [5]Department of Rehabilitation Medicine and Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA, United States, [6]Bio-Materials and Technology Lab, Grain Science and Industry, Bio and Agricultural Engineering, Kansas State University, Kansas, KS, United States, [7]Cardiovascular Disease AdvenHealth Orland, Orland, FL, United States

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**Edited by:**
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***Correspondence:**
*Yuanyuan Zhang*
*yzhang@wakehealth.edu*

*†These authors have contributed equally to this work and share first authorship*

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Telomerase activity is essential for the self-renewal and potential of embryonic, induced pluripotent, and cancer stem cells, as well as a few somatic stem cells, such as human urine-derived stem cells (USCs). However, it remains unclear how telomerase activity affects the regeneration potential of somatic stem cells. The objective of this study was to determine the regenerative significance of telomerase activity, particularly to retain cell surface marker expression, multipotent differentiation capability, chromosomal stability, and *in vivo* tumorigenic transformation, in each clonal population of human primary USCs. In total, 117 USC specimens from 10 healthy male adults (25–57 years of age) were obtained. Polymerase chain reaction amplification of a telomeric repeat was used to detect USCs with positive telomerase activity ($USCs^{TA+}$). A total of 80 $USCs^{TA+}$ (70.2%) were identified from 117 USC clones, but they were not detected in the paired normal bladder smooth muscle cell and bone marrow stromal cell specimens. In the 20–40 years age group, approximately 75% of USC clones displayed positive telomerase activity, whereas in the 50 years age group, 59.2% of the USC clones expressed positive telomerase activity. $USCs^{TA+}$ extended to passage 16, underwent 62.0 ± 4.8 population doublings, produced more cells, and were superior for osteogenic, myogenic, and uroepithelial differentiation compared to $USCs^{TA-}$. Importantly, USCs displayed normal chromosome and no oncological transformation after being implanted *in vivo*. Overall, as a safe cell source, telomerase-positive USCs have a robust regenerative potential in cell proliferation and multipotent differentiation capacity.

**Keywords: telomerase, urine-derived stem cells, longevity, tissue regeneration, differentiation**

## 1 INTRODUCTION

Telomerase activity (TA) is closely related to the longevity of pluripotent stem cells (Huang et al., 2014; Li et al., 2020), embryonic stem cells (ESCs) (Hiyama and Hiyama, 2007), induced pluripotent stem cells (iPSCs) (Wang et al., 2012), and tumor cells (Hiyama and Hiyama, 2007). In normal somatic cells, the activity of telomerase extends telomeric repeats and is usually reduced after birth.

Interestingly, TA often cannot be detected in most human mesenchymal stem cells (MSCs) (Zimmermann et al., 2003; Hiyama and Hiyama, 2007), whereas low levels of telomerase are identified in some somatic stem cells from the hematopoietic system (Thongon et al., 2021), intestinal mucosa, and epidermal basal layers (Hiyama and Hiyama, 2007). Human MSCs, such as bone marrow-derived stem cells (BMSCs) (Bernardo et al., 2007; Hiyama and Hiyama, 2007), adipose-derived stem cells (ASCs) (Nava et al., 2015b), and skeletal muscle progenitor cells (SMPCs), often display telomerase negativity, although these stem cells have the MSC phenotype ($SH2^+$, $SH3^+$, $SH4^+$, $CD29^+$, $CD44^+$, $CD14^-$, $CD34^-$, and $CD45^-$) and can differentiate into adipocytes, chondrocytes, and osteoblasts (Zimmermann et al., 2003). A possible reason for the negative telomerase result is the occurrence of alternative lengthening of telomeres (ALT) in MSCs, which is an alternative telomere length-maintaining mechanism (Lafferty-Whyte et al., 2009).

Our previous study was the first to demonstrate that progenitor/stem cells exist in the urine, thus we proposed the name urine-derived stem cells (USCs) (Zhang et al., 2008; Bodin et al., 2010; Wu et al., 2011; Bharadwaj et al., 2013). These cells can be easily isolated from urine samples via a non-invasive approach (Kang et al., 2015), which offers clear advantages over the stem cells harvested from other sources, like bone marrow or adipose aspirates. Clonal USC populations can be readily generated from a single cell by limiting dilution of the starting mixed culture. Each micro-colony will proliferate into a clonal population with many cells (approximately $6.4 \times 10^7$ cells) within 4 weeks. Urine collected over 24 h from one individual will generate approximately 140 clones (or 10 clones/200 mL urine), which will expand to greater than $1 \times 10^8$ cells by passage three within 3 weeks. Cells do not require tissue dissociation procedures with digestive enzymes, which help to preserve cell viability (Lang et al., 2013). Despite their proliferative capacity, USCs display low expression of gene markers of stem cells (SOX2, OCT3/4, c-MYC, and KLF4) (Bharadwaj et al., 2013) whereas providing a robust proliferative potential reaching up to p16 in a >60-day culture. Understanding the impact of TA on cellular proliferation *in vitro* and the potential regenerative capacity *in vivo* is essential to evaluate the suitability of using USCs for tissue repair, disease modeling, and drug development.

It is well known that telomerase activity is critical for the self-renewal or cell proliferation capacity in stem cells, however, the role of TA in multiple differentiation potential is controversial. The aim of this study was to determine the role of TA in maintaining stemness with cell longevity, proliferation capacity, multipotent differentiation potential, cell surface marker expression, karyotype stability, and the risk of *in vivo* teratoma formation in human primary urine-derived stem cells (USCs).

# 2 MATERIALS AND METHODS

## 2.1 Collection and Culture of Urine-Derived Stem Cells

This study was approval by the Wake Forest University Health Sciences Institutional Review Board. A total of 50 urine samples were collected from 10 healthy male individuals ranging from 25 to 57 years of age and divided into four age groups (20, 30, 40, 50 years of age). A total of 117 USCs clones were isolated, expanded, and characterized as previously described (Zhang et al., 2008; Bodin et al., 2010; Wu et al., 2011). Briefly, USCs were grown in culture media composed of keratinocyte serum-free medium (KSFM) and embryonic fibroblast medium (EFM) mixed at a ratio of 1:1 (Zhang et al., 2008). Only wells in 24 plates that contained single cell were scored and used for further experimentation.

## 2.2 Telomerase Activity Assay

Telomerase activity levels were measured with the Telo *TAGGG* Telomerase PCR ELISA plus kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's recommendations. HEK 293 cells were used as a positive control, and human BMSC and smooth muscle cells (SMC) were used as negative controls. Briefly, $2 \times 10^5$ cells at passage two were collected after being trypsinized and washed with cold PBS. Telomerase added the telomeric repeats (TTAGGG) in the kit to the 3' end of the biotin-labeled synthetic P1-TS primer. These elongated products, and the internal standard (IS) included in the same reaction vessel, were amplified by PCR using the primers P1-TS and the anchor-primer P2. The PCR products were divided into two aliquots, denatured, and hybridized separately to digoxigenin (DIG) labeled detection probes specific for the telomeric repeats and for IS (P3-Std). The resulting products were immobilized via the biotin label onto a streptavidin-coated microplate. Immobilized amplicons were detected with an antibody against digoxigenin that is conjugated to horseradish peroxidase (Anti-DIG-HRP) and the sensitive peroxidase substrate TMB. Absorbance values were measured as the $A_{450nm}$ reading against a blank (reference wavelength $A_{690nm}$) by using a spectrophotometer. Relative telomerase activity (RTA) within different samples in an experiment were obtained using the following formula (Kim et al., 1994; Kim and Wu, 1997):RTA = [(AS–AS, O)/AS, IS]/[(ATS, 8–ATS8, 0)/ATS8, IS]] × 100.

AS: absorbance of sample; AS, 0: absorbance of heat-treated sample; AS, IS: absorbance of Internal standard (IS) of the sample; ATS8: absorbance of control template (TS8); ATS8, 0: absorbance of lysis buffer; ATS8, IS: absorbance of Internal standard (IS) of the control template (TS8). The kit included the IS and TS8.

To further determine the influence of time on telomerase activity, the RTA of two pairs of USCs (age group 20–50 years), telomerase activity positive ($TA^+$) and telomerase activity negative ($TA^-$), after every five passages or the end passage were measured by the above protocol (Kim et al., 1994).

## 2.3 Cell Proliferation

The USCs in passage three were seeded in 96-well plates at a density of 2,500 cells/well. The culture medium was replaced every second day. Cell proliferation was determined on days 1, 3, 5, and 7 using an MTS cell proliferation assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) according to the manufacturer's instructions. Briefly, the MTS reagent was incubated with the cells in the dark for 1 h at 37°C.

Following incubation, the absorbance was measured at 490 nm using a spectrophotometer (Molecular Devices Inc., Sunnyvale, CA, United States ). Triplicate measurements were conducted for each time point. The population doubling (PD) and doubling time (DT) calculations were determined based on single USCs at p0 up to the maximum passage (p16) for each clone. The USCs were trypsinized when they reached 70–80% confluence, and the cells were counted manually using a hemocytometer. The PD and DT were calculated using the following formula (Bharadwaj et al., 2011; Bharadwaj et al., 2013):

$PD = \ln (N_f/N_i)/\ln (2)$ and $DT = C_t/PD$

$N_f$ is the final number of cells, $N_i$ is the initial number of cells, and $C_t$ is the culture time.

## 2.4 Flow Cytometry

Flow cytometry analysis for the USCs involved staining the USCs with specific labeled anti-human antibodies: CD25-PE, CD31-FITC, CD34-FITC, CD44-FITC, CD45-FITC, CD73-PE, CD90-FITC, CD105-PerCP-CY5.5, CD117-PE, CD140b-PE, and CD146-PE (BD Pharmingen™). Briefly, USCs (p4) were trypsinized, and $5.0 \times 10^5$ cells were washed with pre-chilled PBS containing 1% bovine serum albumin (BSA). The fluorescence conjugated antibodies listed above were incubated with USCs on ice for 30 min in the dark. IgG1-PE, IgG1-FITC, IgG2b-FITC, and IgG1-PerCP-Cy™5.5-conjugated isotype control antibodies were used to determine background fluorescence. Cells were washed twice with wash buffer, passed through a 70 μm filter, and analyzed using FACSCalibur™ flow cytometry (BD Biosciences, Franklin Lakes, NJ, United States).

## 2.5 Multipotent Differentiation of USCs *In Vitro*

To determine the differentiation capacity difference between $USCs^{TA+}$ and $USCs^{TA-}$, cells were subjected to the following induction described below, and their changes in morphology and/or histochemical staining for specific components were recorded.

Smooth muscle cell induction: Three pairs of $USCs^{TA+}$ and $USCs^{TA-}$ (p3) from different age groups were plated at 2,000 cells/$cm^2$ in smooth muscle differentiation media containing equal amounts of DMEM (high glucose) and EFM with 10% fetal bovine serum (FBS) and 2.5 ng/mL TGF-β1 and 5 ng/mL PDGF-BB (R&D Systems, Minneapolis, MN, United States). Cell morphology was recorded before and after growth factor additions for up to 14 days.

Uroepithelial induction: Three pairs of $USCs^{TA+}$ and $USCs^{TA-}$ (p3) were plated at 3,000 cells/$cm^2$ in a medium containing equal amounts of KSFM and EFM with 2% FBS and 30 ng/mL EGF (R&D Systems, Minneapolis, MN, United States ) mixed with the conditional medium of urothelial cells (UC, 1:1) for 14 days.

Osteogenic induction: USCs (p3) were seeded at a density of 4,000 cells/$cm^2$ and cultured in serum containing DMEM low-glucose medium with 100 nM dexamethasone, 10 mM β-glycerophosphate and 50 mM ascorbic acid-2-phosphate (Wako Chemicals, Richmond, VA, United States ) for 28 days. The induced cells were harvested after 28 days and fixed in 95% ethanol before histochemical staining. For detection of calcium secreted by the osteogenic-differentiated cells, Alizarin Red S staining was conducted. Briefly, the fixed cells were incubated with 0.5% Alizarin Red S dye (pH 4.1) to sufficiently cover the cell layer for 3–5 mins. Excess dye was removed with distilled water before photo documentation.

## 2.6 Western Blotting

Proteins of SMC and UC-induced $USCs^{TA+}$ and $USCs^{TA-}$ and were extracted using RIPA lysis buffer with a proteinase inhibitor cocktail (Complete mini; Roche Applied Sciences). Protein samples (15–30 μg) were analyzed on a 6–12% SDS-PAGE, and after electrophoresis the proteins were transferred to PVDF membranes (Millipore, Billerica, MA, United States ). The membranes were blocked with skimmed milk and incubated with the primary antibody at an appropriate dilution overnight at 4°C. After secondary antibody incubation, hybridization was detected using the Western Lightning Chemiluminescence reagent (PE, Waltham, MA, United States).

## 2.7 Permeability Assay

To assess the barrier function of urothelial-differentiated $USCs^{TA+}$ and $USCs^{TA-}$, non-induced $USCs^{TA+}$ and $USCs^{TA-}$, SMCs, and ureter UCs, these cells were cultured on 0.4 μm Transwell inserts (353,090, Becton Dickinson) placed in 6-well dishes as previously reported (Cross et al., 2005) with minor modifications. Briefly, the inserts were coated with collagen IV (3 μg/$cm^2$) and air-dried in a laminar hood. Cells ($1 \times 10^5$/$cm^2$) were plated in 1.5 mL of 1 mg/mL tracer-containing medium (FITC-dextran, 4 kDa, Sigma, FD4) in the insert (top chamber) and 3 mL tracer-free medium in the bottom well. Phenol-free medium was used to avoid interference from the indicator in the assay. A day before the tracer was added, the media were supplemented with 2 mM $CaCl_2$ solution. After 3 h, 100 μL media aliquots were removed for fluorescence measurements (excitation at 490 nm and emission at 520 nm). Results were plotted as a percentage relative to the "no cell" control.

## 2.8 Karyotype Analysis

To determine the chromosomal stability of cultured USCs, the karyotypes of early (p4) and late passage ($TA^+$ clone at p12, $TA^-$ clone at p8) cells were measured (Ge et al., 2011). Cultured cells were trypsinized with a 0.25% Trypsin-EDTA solution, resuspended in hypotonic solution (0.075 M KCl) and then fixed with methanol/acetic acid solution in a 3:1 proportion. The metaphase spread on glass slides was digested by trypsin and then stained with Giemsa stain to generate G bands along each chromosome. Standard cytogenetic analysis was performed under microscopy. Chromosomal image capture and karyotyping were performed using CytoVision®, version 3.7.

## 2.9 Soft Agar Assay in Vitro

Agar assays are often used to distinguish tumor cells from non-transformed or normal cells because normal cells cannot undergo anchorage-independent growth or thrive on an agar substrate. To evaluate whether both $USCs^{TA+}$ and $USCs^{TA-}$ induce tumorigenicity, USCs were tested on agar gels. HeLa cells and SMC were used as positive and negative controls, respectively.

Briefly, 0.35% upper agar layer and 4% base agar layer were prepared in 35 mm tissue-treated dishes. Cells were seeded at the top of the upper agar at a density of 5,000 cells/well. Culture medium was changed twice a week. Cell morphology, proliferation rate, anchorage-independent growth, and cell colony formation were observed under a phase contrast microscope. After culturing for 2 weeks, all the samples were stained with 1 mL of 0.05% nitroblue tetrazolium (NBT) prepared in PBS and sterilized (0.2 micron). This was incubated overnight at 37°C. The cells that took up NBT and showed a violet color were determined to be live cells.

## 2.10 Spontaneous Transformation *In Vivo* Assay

Experiments using nude mice were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee. To further determine the non-tumorigenicity of USCs$^{TA+}$ *in vivo*, two USCs$^{TA+}$ (p5) were implanted in the kidney subcapsular region of NSG (NOD.Cg-Prkdcscid-Il2rgtm1Wjl/SzJ) mice. H9 (human embryonic stem cell line) was used as a positive control. A total of 12 six-week-old female NSG mice (Jackson Labs, Bar Harbor) were used and divided into two groups: USC$^{TA+}$ (3 mice/clone) and H9 groups (6 mice/group). In total, $2 \times 10^6$ cells in 20 µl of PBS were injected into the subcapsular region of the right kidney. After 8 weeks, the mice were sacrificed and bilateral kidneys were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. For evaluation of general graft histology and teratoma formation, routine hematoxylin and eosin (H&E) staining was performed.

# 3 RESULTS

## 3.1 Telomerase Activity Detected in USCs at Early Passage

A total of 80/117 USC clones (p2) from 10 individuals showed detectable TA (70.2% were USCs$^{TA+}$). There were no significant differences in the positive rate for TA in the 20–40 years age group: 20 s: 30/39 (76.9%), 30s: 14/19 (73.7%), and 40s: 16/22 (72.7%) represented USC clones, respectively, but the RTA notably decreased to 16/27 (59.2%) in the 50 years age group, as shown in **Figure 1** and **Table 1**. TA could not be detected in human BMSCs at p2.

To determine if the TA in human USCs decreased as the passage progressed, we measured the TA of two pairs of USCs$^{TA+}$ and USCs$^{TA-}$ at early passage (p2) and late passages (p7, p11 or the last passage for USCs$^{TA+}$) from the 20s and 50s groups. The strength of RTA of USC$^{TA+}$ clones decreased gradually with passage (**Figure 2**), which seems to make a formerly TA+ clone equivalent to a TA$^-$clone. The RTA remained at undetectable levels through all the passages of USC$^{TA-}$ clones.

## 3.2 Self-Renewal Ability of USCs$^{TA+}$ Generated More Cells

The USC$^{TA+}$ and USCs$^{TA-}$ paired clones from a single donor were tested in parallel to prevent other factors from affecting the results of the comparison. The USC$^{TA+}$ clone A35 which was isolated from a 27-year-old male donor, could be passed more than the USCs$^{TA-}$ clone A42 which was isolated from the same donor, and consistently maintained the original "rice-grain like" morphology until they reached cell senescence at p16. Similarly, the USC$^{TA-}$ clones steadily displayed the similar cell morphologic appearances and finally displayed a larger, flattened, and the typical "fried egg" morphology of quiescent cells at p9 (**Figure 3**).

According to the cell growth curve data (**Figure 4**), the USC$^{TA+}$ clones grew more rapidly than the USC$^{TA-}$ clones. Consequently, the PD of USC$^{TA+}$ clones increased significantly compared to that of the USC$^{TA-}$ clones, regardless of the individual's age ($p < 0.001$). The mean DTs of USC$^{TA+}$ clones were significantly shorter than those of the USC$^{TA-}$ clones (**Table 2**; **Figure 5**) ($p < 0.001$).

However, the relative TA of USCs was not completely coordinated with the cell proliferation capacity (PD, DT, and passage) (**Table 2**).

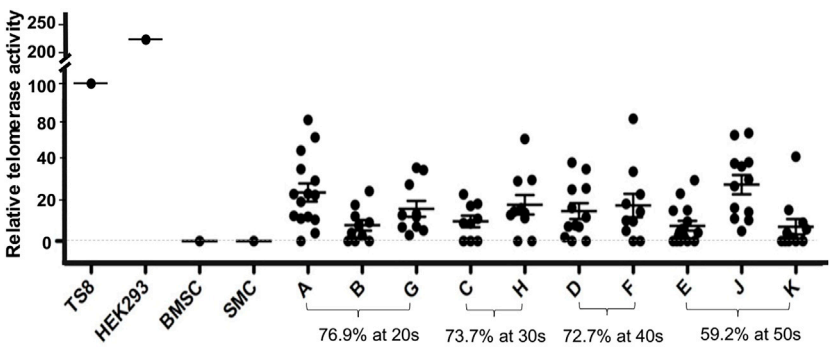


**FIGURE 1 |** Distribution of human USCs$^{TA+}$ in different age groups. Most USCs (76.9–72.7%) retained telomerase activity in donors at age 20–40 years of age, but the ratio of USCs$^{TA+}$ significantly dropped to 59.2% in the donors over 50 years. Cell extracts were prepared from $2 \times 10^5$ cells and assayed for telomerase activity, according to the manufacturer's instructions (Telo *TAAGG* ELISAPLUS kit Roche). TS8 was provided as a representative positive control lysate prepared from HEK-293 cells. BMSC; human mesenchymal stromal cells of bone marrow, SMC; smooth muscle cells as controls, and A, B, C, D, E, F, G, H, J, K; individual USC clones from different aged healthy individuals (20–50 s), at passage 2 (p2). RTA is the percentage (%) of TS8 (internal positive control) after being normalized.

**TABLE 1 |** The rate, mean, and highest telomerase activity of the isolated USCs$^{TA+}$ clones for individual donors (p2) in the four different age groups.

| Donor's ages (no. of donors) | Donors (no. of clones) | No. of USCTA + clones (% in total USC | Mean TA ± SD (%) | Highest RTA |
|---|---|---|---|---|
| 20s (*n* = 3) | A (*n* = 19) | 16 (84.2%) | 24.38 ± 14.41 | 53.25 |
| | B (*n* = 10) | 6 (60.0%) | 14.94 ± 7.93 | 25.94 |
| | G (*n* = 10) | 8 (80.0%) 76.9% | 16.85 ± 10.86 | 32.24 |
| 30s (*n* = 2) | C (*n* = 9) | 6 (66.6%) | 12.98 ± 5.28 | 20.56 |
| | H (*n* = 10) | 8 (80.0%) 73.7% | 19.94 ± 12.05 | 44.93 |
| 40s (*n* = 2) | D (*n* = 12) | 9 (75.0%) | 17.41 ± 10.99 | 34.50 |
| | F (*n* = 10) | 7 (70.0%) 72.7% | 21.69 ± 16.03 | 53.75 |
| 50s (*n* = 3) | E (*n* = 17) | 7 (41.2%) | 18.55 ± 12.37 | 41.36 |
| | J (*n* = 10) | 9 (90.0%) | 26.67 ± 13.84 | 47.49 |
| | K (*n* = 10) | 4 (40.0%) 59.2% | 16.03 ± 14.50 | 37.12 |

*Notes: Telomerase activity was expressed as a percentage of the RTA, of USCs, to the RTA, of TS8. The suffixes A, B, G, C, H, D, E, F, J, K represent the ten healthy individuals who participated in the study.*

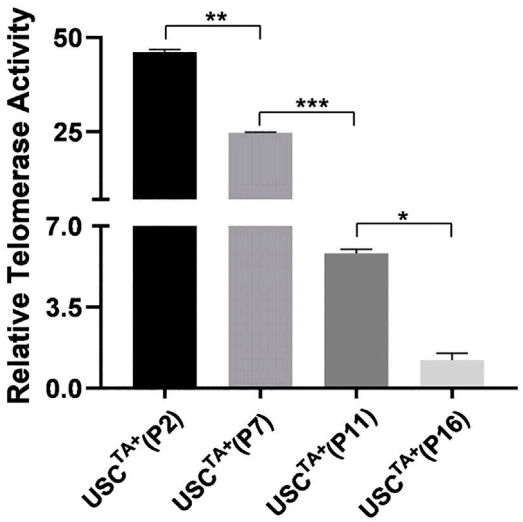


**FIGURE 2 |** Strength of telomerase activity declined over passage of cultured USCs. Telomerase activity of USC$^{TA+}$ clones (A35 and J29) from donors of 20 and 50 years of age were determined in different passages (p2, p7, p11, and the last passage). The relative telomerase activity (RTA) of USC$^{TA+}$ clones declined gradually in the passages during culture *in vitro*.

## 3.3 Both USC$^{TA+}$ and USC$^{TA-}$ Clones Showed Similar Cell Surface Markers

To identify the cell surface markers of USC clones, six pairs of USCs$^{TA+}$ and USCs$^{TA-}$ were subjected to flow cytometry analysis. All the USC clones showed strong positive MSCs markers including CD44, CD90, CD73, CD105, and CD146 and were negative for hematopoietic stem cell markers including CD25, CD31, CD34, CD45, and CD117 (**Figure 6**; **Table 3**). However, there was no significant difference in the CD105 expression between USCs$^{TA+}$ and USCs$^{TA-}$.

## 3.4 Potent Differentiation Capacity of USCs$^{TA+}$

The USC$^{TA+}$ and USCs$^{TA-}$ paired clones from a single donor were tested in parallel to prevent other factors from affecting the results of the comparison. To test the difference in differentiation capability between USCs$^{TA+}$ and USCs$^{TA-}$, both USCs$^{TA+}$ and USCs$^{TA-}$ (p3) were induced to osteogenic, myogenic, and uroepithelial differentiation (**Table 4**). Both the USCs$^{TA+}$ clone A35 that was isolated from a 27 year-old male donor, and the USCs$^{TA-}$ clone A42 that was isolated from the same donor (p3) differentiated into the smooth muscle lineage and the urothelial lineage (**Figures 7A,B**). Urothelial-differentiated cells developed a cobblestone-like morphology (**Figure 7Ai**) and expressed the urothelial-specific proteins uroplakin-Ia and uroplakin-III and the generic epithelial cell markers CK7, CK13, and AE1/AE3 (**Figure 7Aii**). Furthermore, the expression of these proteins was significantly higher in the UC-induced USCs$^{TA+}$ clones [A35 and J29 (from another male aged 55 years)] than in the UC-induced USCs$^{TA-}$ clones (A42 and J18 [from the same male aged 55 years as clone J29]) and uninduced USC clones. In an assay of cellular barrier function, the UC-induced USCs$^{TA+}$ clone-A35 showed increased expression of specific tight junction protein markers (E-cadherin) compared to the UC-induced USCs$^{TA-}$clone-A42 and uninduced USC clones (**Figure 7Ai**). In barrier function assays, both the urothelial-differentiated USCs (USC$^{TA+}$ 41.0% ± 1.7% and USC$^{TA-}$ 44.8% ± 0.4%) showed significant reduction in leakage of fluorescent tracer through the insert *in vitro* ($p < 0.01$ and $p < 0.001$, respectively), which means lower permeability and higher tight junction property, compared to the non-differentiated USCs (USC$^{TA+}$ 63.1% ± 4.3%, USC$^{TA-}$ 62.2% ± 1.1%) (**Figure 7Aiii**) at day 3; and similar results received at day 7 ($p < 0.01$), the leakage percentage of urothelial-differentiated USCs (USC$^{TA+}$-A35 35.5% ± 1.2% and USC$^{TA-}$-A42 38.9% ± 4.3%), less than the non-differentiated USCs (USC$^{TA+}$-A35 54.8% ± 2.6%, USC$^{TA-}$-A42 59.6% ± 0.5%). However, there was only a slight difference in leakage protection at day 3 between the UC-induced USC$^{TA+}$-A35 and USC$^{TA-}$-A42 clones ($p < 0.05$). In addition, no significant differences between them, although reduce in leakage, were noted in UC-induced USC$^{TA+}$-A35 clone on day 7.

Both myogenic differentiated USCs$^{TA+}$ and USCs$^{TA-}$ became elongated and spindle-shaped (**Figure 7Bi**) and expressed smoothelin, a smooth muscle-specific protein marker, and calponin (**Figure 7Bii**). Moreover, the expression of

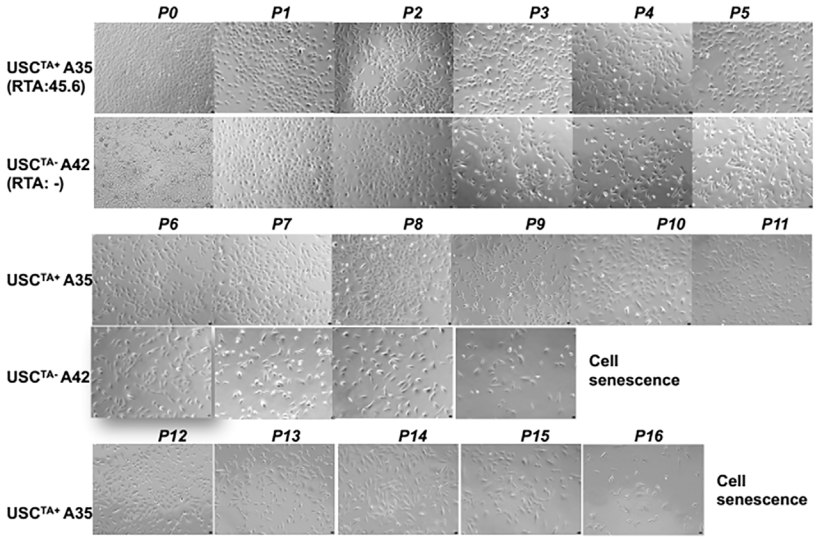


**FIGURE 3 |** Changes in the morphologic appearance of USCs$^{TA+}$ and USCs$^{TA-}$ at different passages. A USC$^{TA+}$ clone (A35, RTA: 45.6, from the donor at 20 years of age) maintained the "rice-grain"-like appearance up to p16; a USC$^{TA-}$ clone (A42, RTA: negative, from the same donor at an age of 20 years of age) showed a flattened cell shape or cells of larger size, and stopped growing (cell senescence) at p9, assessed by phase contrast microscopy. Magnification, ×100.

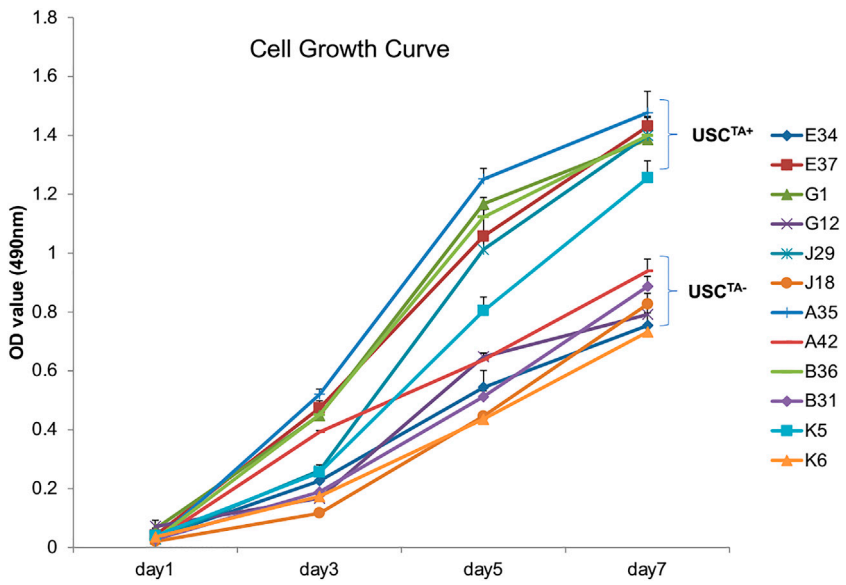


**FIGURE 4 |** Cell growth curve of USCs$^{TA+}$ vs. USCs$^{TA-}$. Cell proliferation was measured as the USCs were cultured for 7 days. Six individual USC$^{TA+}$ clones ($n = 6$, p3, A35, B36, G1, E37, J29, and K5) generated significantly more cells and grew faster than USC$^{TA-}$ clones ($n = 6$, p3, A42, B31, G12, E34, J18, and K6).

smoothelin and calponin of SMC- differentiated USCs$^{TA+}$-A35 were higher than the SMC-differentiated USCs$^{TA-}$-A42 confirmed by western blotting (**Figure 7Bii**). Finally, both USCs$^{TA+}$-A35 and USCs$^{TA-}$-A42 were also induced to differentiate into the osteogenic lineage using our previous protocols. USCs$^{TA+}$-A35 could be induced to osteocytes but not USCs$^{TA-}$-A42, as confirmed by Alizarin S Red staining (**Figure 7C**). Moreover, both USCs$^{TA+}$-A35 and USCs$^{TA-}$-A42 were difficult to differentiate into adipocytes, as evidenced by Oil Red-O staining (data not shown).

## 3.5 Karyotype Remains Stable in USCs$^{TA+}$

To investigate the potential susceptibility of USCs$^{TA+}$ to malignant transformation, cells were tested via cytogenetic analysis, agar culture *in vitro*, and teratoma formation *in vivo*, and the results were compared to those of USCs$^{TA-}$ and controls.

**TABLE 2 |** Population doubling and doubling time of $USC^{TA+}$ vs $USC^{TA-}$ in the 20 and 50 years age groups in early and late passages to determine if there was a difference in telomerase activity with increasing age.

| Individual clones (s) | | | Age (yrs) | RTA (%) | Population Doubling | | Doubling Time | |
|---|---|---|---|---|---|---|---|---|
| | | | | | PD | M ± SD | DT (hrs.) | M ± SD |
| 20s | $USC^{TA+}$ (n = 3) | A35 | 27 | 45.6 | 67.5 | 62.0 ± 4.8* | 27.1 | 26.1 ± 1.6** |
| | | G1 | 25 | 25.9 | 59.2 | — | 24.3 | — |
| | | B36 | 28 | 32.2 | 59.2 | — | 27.0 | — |
| | $USC^{TA-}$(n = 3) | A42 | 27 | — | 42.0 | 39.6 ± 2.6 | 37.3 | 35.2 ± 2.4 |
| | | G12 | 25 | — | 36.8 | — | 35.7 | — |
| | | B31 | 28 | — | 40.1 | — | 32.7 | — |
| 50s | $USC^{TA+}$ (n = 3) | E37 | 50 | 41.4 | 55.5 | 56.6 ± 2.2* | 29.2 | 28.5 ± 1.3** |
| | | J29 | 55 | 46.6 | 59.2 | — | 27.0 | — |
| | | K5 | 57 | 37.1 | 55.2 | — | 29.3 | — |
| | $USC^{TA-}$(n = 3) | E34 | 50 | — | 35.6 | 36.3 ± 0.9 | 36.6 | 36.6 ± 2.5 |
| | | J18 | 55 | — | 36.2 | — | 34.2 | — |
| | | K6 | 57 | — | 37.2 | — | 39.1 | — |

*p < *0.001: $USC^{TA+}$ vs $USC^{TA-}$ in, population doubling at age of 20s, and 50s groups, respectively.*

**p < 0.01*: $USC^{TA+}$ vs $USC^{TA-}$ in, doubling time at age of 20s, and 50s groups, respectively. The suffixes A, B, G, C, H, D, E, F, J, and K represent the ten healthy individuals who participated in the study.*

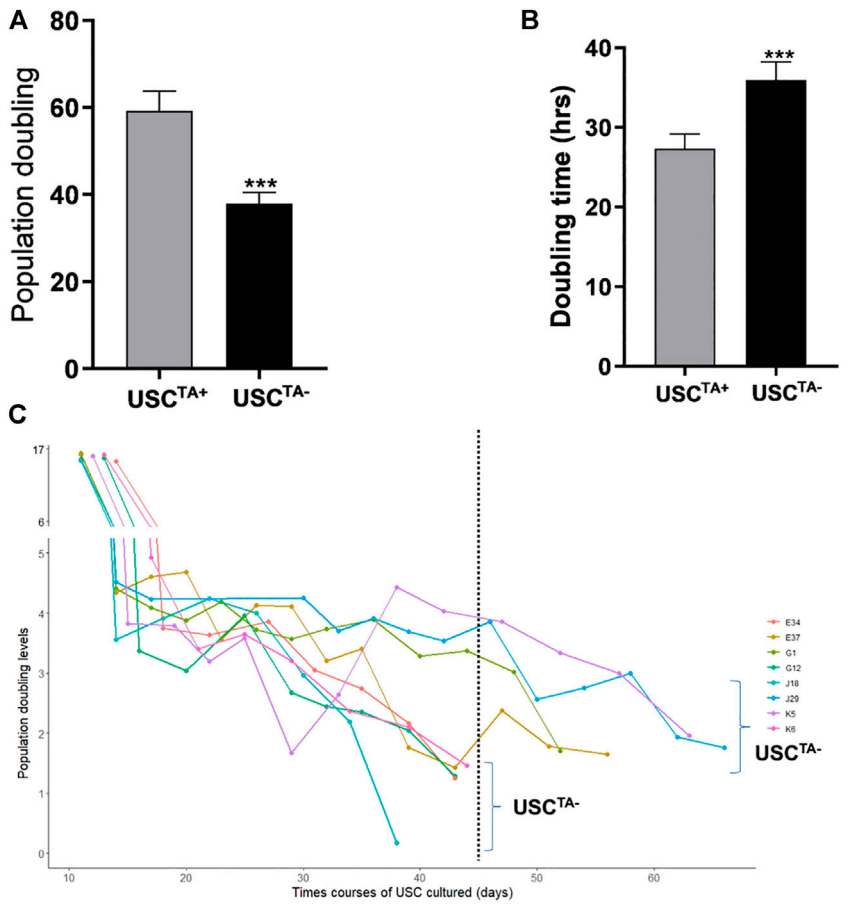


**FIGURE 5 |** Population doubling, doubling time, and *in vitro* survival time course of $USCs^{TA+}$ vs $USCs^{TA-}$. **(A)** Population doubling significantly increased for $USCs^{TA+}$ compared to $USCs^{TA-}$. **(B)** Doubling time was significantly shorter in $USCs^{TA+}$ than in $USCs^{TA-}$. **(C)** Cellular senescence in $USCs^{TA-}$ occurred earlier than that in $USCs^{TA+}$ upon cell expansion. These data indicate that $USCs^{TA+}$ proliferates more rapidly, generates more cells, and survives longer than $USCs^{TA-}$. USCs from healthy individuals ($n$ = 12) were cultured following plating at a single cell/well. Six individual $USC^{TA+}$ clones ($n$ = 6, p3, A35, B36, G1, E37, J29, and K5) generated significantly more cells and grew faster than $USC^{TA-}$ clones ($n$ = 6, p3, A42, B31, G12, E34, J18, and K6). $USC^{TA+}$ clones survived longer and had longer population doubling times. The suffixes A, B, G, C, H, D, E, F, J, and K represent the ten healthy individuals who participated in the study.

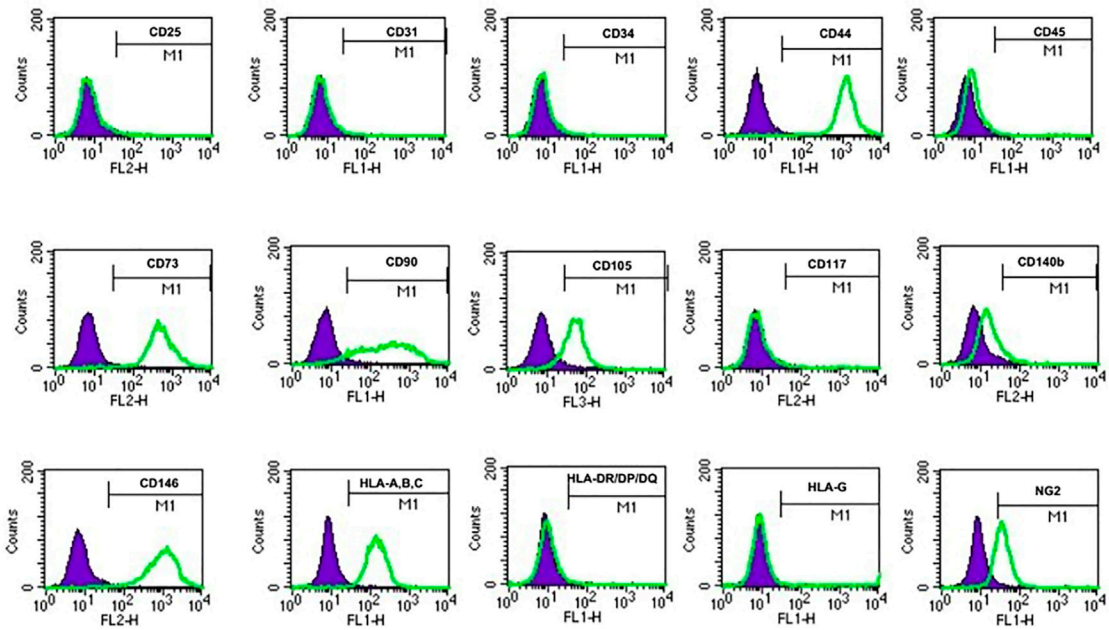


**FIGURE 6 |** USCs expressing mesenchymal stem cell surface markers. USC$^{TA+}$ clones (A35, B36, G1, E37, J29, and K5) and USC$^{TA-}$ clones (A42, B31, G12, E34, J18, and K6) both displayed sets of mesenchymal stem cell (MSC) surface markers (CD44, CD90, CD73, CD105, and CD146), positive at similar levels, whereas both USCs$^{TA+}$ and USCs$^{TA-}$ did not express sets of haemopoietic stem cell markers (i.e., CD31, CD34, CD45, CD25, and CD117). However, three of five USCs$^{TA+}$ displayed CD105 expression, but no or weak expression in all the four USC$^{TA-}$ cell clones. MSC surface markers of USCs at p4 were detected *via* flow cytometry.

**TABLE 3 |** Cell surface markers of USC$^{TA+}$ and USC$^{TA-}$ clones at passage four detected using fluorescence-activated cell sorting.

| CLONES (s) | | | Cell surface markers (%) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | CD 25 | CD 31 | CD 34 | CD 44 | CD 45 | CD 73 | CD 90 | CD 105 | CD 117 | CD 140b | CD 146 | NG2 | HLA-a,b,c | HLA-DR/DP/DQ | HLA-G |
| **20s** | TA+ | A35 | 0.36 | 0.51 | 0.40 | 99.9 | 0.34 | 99.8 | 93.4 | **82.2** | 0.5 | 3.9 | 99.89 | 3.05 | 99.97 | 0.81 | 4.53 |
| | TA– | A42 | 0.69 | 0.81 | 0.98 | 100 | 0.54 | 99.9 | 95.1 | **5.3** | 0.6 | 7.7 | 99.86 | 29.34 | 99.98 | 1.18 | 0.30 |
| | TA+ | G1 | 0.37 | 0.09 | 0.07 | 100 | 0.09 | 99.86 | 90.5 | **90.7** | 0.4 | 5.0 | 99.96 | 88.56 | — | — | — |
| | TA– | G12 | 0.04 | 0.39 | 0.20 | 99.7 | 0.02 | 97.70 | 99.8 | **99.0** | 0.9 | 0.1 | 99.67 | 0.44 | — | — | — |
| | TA+ | B36 | 0.21 | 0.52 | 0.60 | 96.8 | 0.77 | 99.73 | 99.6 | **33.0** | 0.8 | 12.0 | 99.08 | 15.48 | 99.69 | 0.67 | 0.86 |
| | TA– | B31 | 0.60 | 0.61 | 0.55 | 99.8 | 0.93 | 99.91 | 99.8 | **1.7** | 0.5 | 9.3 | 99.66 | 29.56 | 99.90 | 0.64 | 0.84 |
| **50s** | TA+ | E37 | 1.37 | 0.86 | 0.52 | 99.8 | 0.98 | 99.97 | 98.9 | **66.1** | 0.93 | 0.1 | 99.26 | 5.00 | — | — | — |
| | TA– | E34 | 3.45 | 0.77 | 0.99 | 100 | 0.70 | 99.98 | 99.7 | **91.4** | 1.41 | 0.2 | 99.76 | 25.20 | — | — | — |
| | TA+ | J29 | 0.41 | 0.33 | 0.16 | 99.94 | 0.37 | 100 | 92.12 | **55.44** | 0.31 | 5.98 | 98.12 | 3.06 | 99.97 | 0.13 | 1.09 |
| | TA– | J18 | 0.45 | 0.55 | 0.53 | 98.36 | 0.41 | 95.61 | 95.61 | **3.50** | 0.44 | 0.62 | 96.51 | 59.35 | 98.40 | 1.06 | 1.73 |
| | TA+ | K5 | 0.46 | 0.33 | 0.26 | 99.85 | 0.35 | 99.90 | 83.20 | **48.67** | 0.41 | 8.71 | 99.14 | 64.55 | 99.70 | 0.48 | 1.90 |
| | TA– | K6 | 0.71 | 0.47 | 0.26 | 99.57 | 0.32 | 89.84 | 49.13 | **15.25** | 0.70 | 8.39 | 99.41 | 71.18 | 99.20 | 0.37 | 1.42 |

**TABLE 4 |** Induced and non-induced multipotent differentiation potential of USCs.

| | Positive control | Induced USC$^{TA+}$ | Non-induced USC$^{TA+}$ | Induced USC$^{TA-}$ | Non-induced USC$^{TA-}$ |
|---|---|---|---|---|---|
| Myogenic differentiation | (4 +) SMC | (3 +) in 1/2 | 0/1 | 0/2 | 0/1 |
| Urothelial differentiation | (4 +) UC | (3 + ~ 4 +) in 2/4 | 0/1 | 0/2 | 0/1 |
| Osteogenic differentiation | (4 +) ASC | (2 + ~ 3 +) in 2/2 | N/A | 0/2 | N/A |

*Abbreviation; SMC, smooth muscle cells; UC, urothelial cells; ASC, adipose-derived stem cells. Notes: 1+, 1–25% differentiated cells; 2+, 26–50% differentiated cells. 3+, 51–75% differentiated cells. 4+, >75% differentiated cells.*

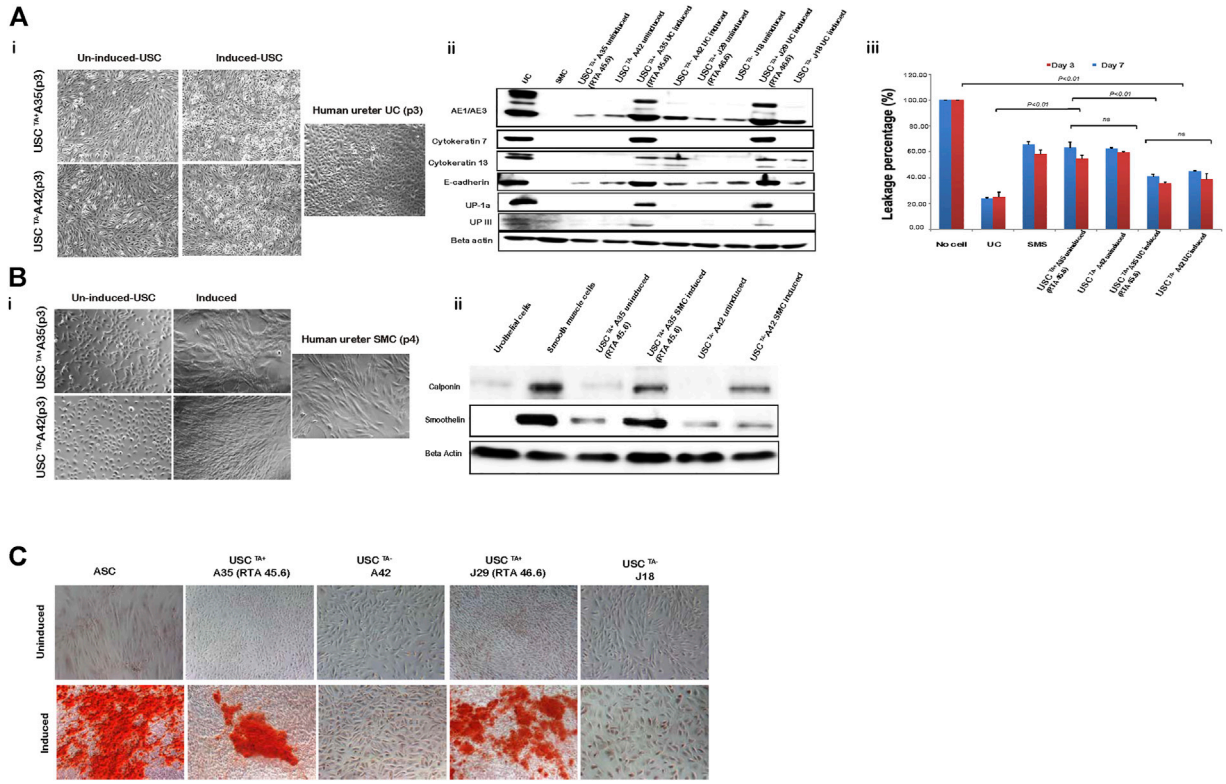


**FIGURE 7 |** USCs undergo multi-potential differentiation *in vitro*. **(A)**. Morphology of USCs after induction with USC induction media for 14 days. Cell morphology changed from "rice-grain-like" to a cuboidal morphology appearance. Human ureter urothelial cells (UC) were included as a positive control. Urothelial-specific proteins (Uroplakin-Ia, -III, AE1/AE3, E-cadherin, Cytokeratin 7, and Cytokeratin 13) were detected via western blotting. Specific signals (bands) were observed in lanes with proteins from induced USCs$^{TA+}$ and UCs. Barrier function analysis was performed on both USCs$^{TA+}$ and USCs$^{TA-}$ differentiated to UC-like cells for 3 and 7 days. FITC-dextran was incubated with cells grown on the insert, and media in the bottom chamber was analyzed after 3 h. Results were plotted as a percentage relative to the "no cell" control. Positive leakage prevention was observed in both urothelial-induced USCs$^{TA+}$ and USCs$^{TA-}$ when compared to the negative control SMCs. **(B)** Morphology of USCs after induction with SMC induction media for 14 days. Cells were spindle-shaped after induction. Human ureter SMCs served as the positive control. Smooth muscle-specific proteins (calponin and smoothelin) were detected via immunoblotting. Specific signals (bands) were observed in lanes with proteins from the induced USCs$^{TA+}$ and SMC control. **(C)** USCs cultured in osteogenic differentiation media were evaluated for osteogenic cell lineages. Osteogenic differentiation-day 21, positive von Kossa staining for bone minerals was observed in the induced USCs$^{TA+}$ and USCs$^{TA-}$ D). Summary of the differentiation capacity of USC$^{TA+}$ vs USC$^{TA-}$ clones. A35-clone (USCs$^{TA+,}$ RTA: 45.60) and A42 clone (USCs$^{TA-}$, RTA: negative) were both isolated from one donor (male, 27 year-old); J29 (USCs$^{TA+}$, RTA:46.6) and J18 (USCs$^{TA-}$, RTA: negative) were isolated from another donor (male, 55 year-old).

Both TA+ and TA- USC clones in the early (p4) and late passage (USCs$^{TA+}$-A35, J29 clone in p12 and USCs$^{TA-}$-A42, J18 clone at p8) displayed a normal karyotype of 1 X and 1 Y chromosome, as expected for a male donor, and a normal diploid (2n = 44) complement of autosomes and a pair of sex chromosomes (**Figure 8A**; **Table 5**). No multiploidy or obvious chromosomal rearrangements in metaphase were detected by Giemsa bandings at p4 or late passage of both USC clones.

## 3.6 No Tumorigenic Transformation of USCs$^{TA+}$ Was Observed

USC$^{TA+}$ clones-A35, J29 remained the same size on day 14 as that on day 1, after being cultured in soft agar, like colonies of SMCs. However, HeLa cancer cells formed large colonies on day 14 (**Figure 8Bi**). These single cells (SMC and USC) or colonies (HeLa cancer cells) were viable, which was confirmed by NBT staining (**Figure 8Bii**). Furthermore, no teratoma was formed when all USCs$^{TA+}$-A35 were implanted in the subcapsular region of the kidney of NSG mice after 8 weeks. All human ES cell lines (H9) formed derivatives of the three embryonic germ layers (**Figure 8C**).

# 4 DISCUSSION

We characterized the stemness features of human USCs, including long-term survival with self-renewal capacity, multi-lineage differentiation, MSCs surface markers, expression of telomere maintenance mechanisms (TA) in *in vitro* culture time frames, and capacity to form teratomas. USC clones in the same individual urine sample displayed telomere heterogeneity, which could be due to USCs at different stages of the telomerase activation processes.

Stemness refers to the molecular processes underlying the fundamental stem cell properties of self-renewal and the

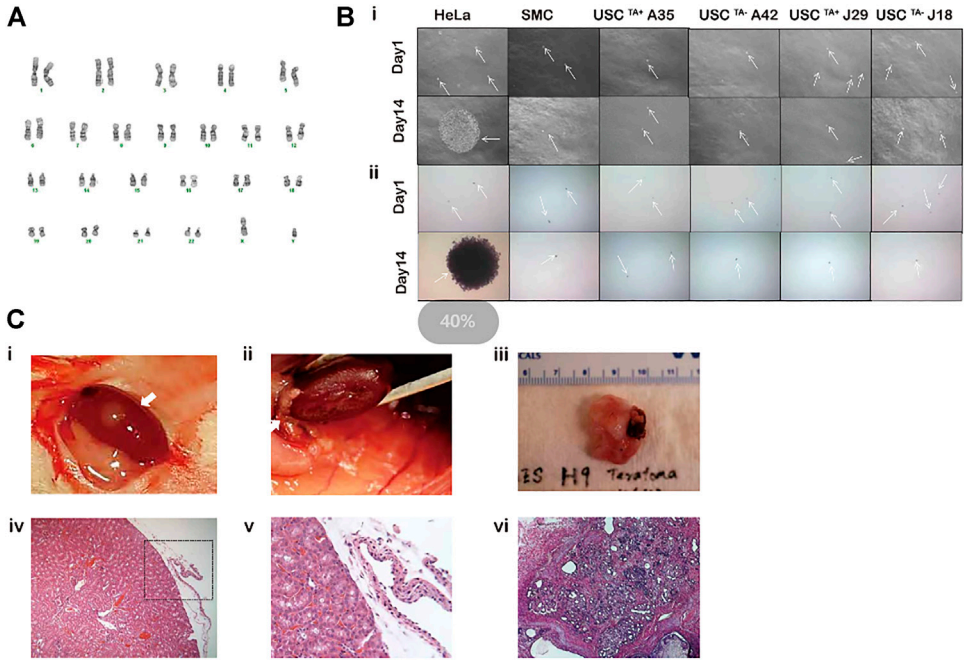


**FIGURE 8 |** Spontaneous transformation assays of USC$^{TA+}$ clones. **(A)** Karyotypes of USC$^{TA+}$ vs. USC$^{TA-}$ clones. Both USC$^{TA+}$ ($n$ = 2, A35, RTA:45.6; and J29, RTA:46.6) and USC$^{TA-}$ ($n$ = 2, A42 and J18) clones in the early passage (p4) and late passage (TA + clone in p12, TA-clone at p8) displayed normal complement of diploid (2n = 46). **(B)** *In vitro* agar assay of USCs. The size of the cell clones (seeded at a density of 5,000 cells/well) of USC$^{TA+}$ clones (A35 and J29) and USC$^{TA-}$ clones (A42 and J18) remained the same between days 1 and 14 after plating on soft agar gel. However, clone size of the cancer cell line HeLa cells, as the positive control, at 14 days were significantly increased compared to that at the first day. Normal bladder smooth muscle cell clones were used as the negative control. Images were captured using a phase contrast microscope. Cell clones were stained with nitro blue tetrazolium and photographed using bright field soft agar assays for anchorage-independent cell growth of USC$^{TA+}$ *in vitro*. **(C)** *In vitro* transformation assay of USCs. No teratoma formation was observed under the microscope 2 months after USC$^{TA+}$ (two million cells/graft, $n$ = 2, white arrow) were implanted into the capsules of the kidneys of NSG mice. There was no teratoma observable grossly (i, ii) or microscopically (iii, iv). In contrast, H9 cells as the positive control formed derivatives of all the three embryonic germ layers (v, vi). A35-clone (USCs$^{TA+}$, RTA: 45.60) and A42 clone (USCs$^{TA-}$, RTA: negative) were both isolated from one donor (male, 27 year-old); J29 (USCs$^{TA+}$, RTA:46.6) and J18 (USCs$^{TA-}$, RTA: negative) were isolated from another donor (male, 55 year-old).

**TABLE 5 |** Conventional karyotypes of USC$^{TA+}$ and USC$^{TA-}$ clones in the 20- and 50-years age groups at early and late passages.

| | Age at 20s group | | Age at 50s group | |
|---|---|---|---|---|
| | **USC clones** | **Karyotypes** | **USC clones** | **Karyotypes** |
| **USC$^{TA+}$** | A35 (p4) | 46, XY | J29 (p4) | 46, XY |
| | A35 (p12) | 46, XY | J29 (p12) | 46, XY |
| **USC$^{TA-}$** | A42 (p4) | 46, XY | J18 (p4) | 46, XY |
| | A42 (p8) | 46, XY | J18 (p8) | 46, XY |

*Notes: A35-clone (USCs$^{TA+}$, RTA: 45.60) and A42 clone (USCs$^{TA-}$, RTA: negative) were both isolated from one donor (male, 27-year-old); J29 (USCs$^{TA+}$, RTA:46.6) and J18 (USCs$^{TA-}$, RTA: negative) were isolated from another donor (male, 55 year-old).*

generation of differentiated daughter cells. The stemness properties of adult-derived stem cells decline after birth, compared to those of ESCs (Hofmeister et al., 2015). Most human somatic or stem cells do not express OCT4/SOX2/KLF4/MYC. Forced expression of OCT4/SOX2/KLF4/MYC in somatic cells such as fibroblasts can reprogram cells to a pluripotent stem cell fate. USCs are multipotent, rather than pluripotent, and express low levels of OCT4/SOX2/KLF4/MYC (Bharadwaj et al., 2013). Our previous studies demonstrated that USCs possess limited stemness properties including self-renewal and multiple differentiation capacity but do not induce teratoma formation. This is different from iPSC that have higher expression levels of OCT4/SOX2/KLF4/MYC and form teratoma. In summary, USCs are multipotent and thus do not express the higher levels of OCT4/SOX2/KLF4/MYC that are observed in pluripotent stem cells.

Two methods are predominantly used to track the *in vitro* age of a cell culture or cell proliferation capacity. 1) The passage number implies the number of times a cell has been passaged, which is most commonly used in the laboratory. However, the cell passage number is imprecise because different laboratories may use different initial cell seeding densities, which affects the number of times cells divide in culture. 2) The PD indicate the number of cell generations the cell lineage has undergone—the number of times the cell population has doubled. PD of primary cells is a better practice for reporting cellular age *in vitro*, which is often used to set an acceptable upper limit for cell production, or the maximum number of cells generated in culture. In this study, we used both terms (passage number and PD number) to present cell lifespan, and there was good agreement between the two measures. USCs$^{TA+}$ with higher TA could reach higher PD number or passage

number, a shorter DT which indicates faster cell division or more rapid cell growth, and better proliferation capacity which indicates more cells generated than those of USCs$^{TA+}$ with lower TA strength and USCs$^{TA-}$. Furthermore, the TA of USCs$^{TA+}$ declined with passage; thus, the proliferation potential with PD and DT of USCs gradually decreased, with the cells finally reaching senescence within 8 weeks in 2D culture and 10 weeks in 3D culture models (data not shown).

Telomerase is activated and maintains cellular immortality in ESCs or iPSCs, which plays an import role such as to protect the genome from nucleolytic degradation, unnecessary recombination, repair, and intrachromosomal fusion (Hiyama and Hiyama, 2007); however, the level of TA is low or absent in most MSCs and ASCs (Zimmermann et al., 2003; Hiyama and Hiyama, 2007) regardless of their proliferative capacity. Numbers of BMSCs are low in bone marrow nucleated cells with a frequency of colony-forming unit-fibroblasts (CFU-F) of 1: 35,700 (Lu et al., 2006). In addition, small amounts of stem cells are mixed with a large amount of stromal cells in the bone marrow, which makes it challenging to isolate true stem cells and measure their levels of TA. In contrast, USCs start with a single stem cell that forms cell clones and expands to a large number of stem cells of which most display TA. There are a couple of studies comparing hUSC to hBMSC (Sun et al., 2021) and ASC (Kang et al., 2015). In *in vitro* experiments, hUSC presented with better capacity for proliferation than hBMSC, while hBMSC had greater chondrogenic ability than hUSC. However, hUSC and hBMSC had similar cartilage repair effects *in vivo*. Results indicated that hUSC can be a stem cell alternative for cartilage regeneration, provide a powerful platform for cartilage tissue engineering, and clinical transformation (Sun et al., 2021). Similar outcomes are achieved in studies of comparison between hUSC and hASC (Kang et al., 2015). TA levels can be detected or consistently expressed in most human USCs (>70%) obtained from healthy middle-aged donors, although levels of this enzyme were lower than those of ESC. Most USCs express TA however, along with the aging process in individuals, a reduction in USC regenerative capacity occurs in the 50s age group, which also means a decrease in cell proliferation capacity along with the reduced number of USCs$^{TA+}$ (59%), and relatively lower PD and DT and a decline in telomere reserve with associated lower TA. Furthermore, the number of USCs$^{TA+}$ declined with increasing passage during cell proliferation. Thus, the levels of TA decreased with an increase in donor age and cell passage but did not show an increase again after long-term culture, demonstrating the safety of USC implantation with retaining chromosome stable and no oncological transformation. The TA of USCs$^{TA+}$ declined with passage *in vitro* or throughout individual age, providing a mechanism that restricts cell over-proliferation and any tumor development.

Human MSCs as a heterogeneous cell population are confirmed by a set of cell surface makers, instead of a single marker. MSCs often maintain their immunophenotypic characteristics stably throughout the culture term. The ASCs did not show immunophenotypic alterations with passage and retained a consistently high expression level of MSC surface markers and were negative for HSC at early (p4) and late passage (p8) markers (Nava et al., 2015a). In this study, we observed there were USCs at different stages of activation. One was the activating USCs with positive telomerase activity; one is activated cells (relatively old) with negative TA; but both USCs$^{TA+}$ and USCs$^{TA-}$ in p4 showed the most MSC surface markers (CD44, CD73, CD90, CD105, CD146, and HLA-A, B, C) and lacked hematopoietic markers (CD25. CD31, CD34, CD45, HLA-DR/DP/DQ, and HLA-G). Our data demonstrated that USCs are a good starting population because of their lack of immunological reactivity. Importantly, through the most exhaustive head-to-head characterization of multiple clones that each start a single USC clonal population from multiple donors, these results allow us to highlight the intrinsic differences between commonly used starting cell sources.

In addition, CD105 is one unique marker associated with differentiation potential (Dominici et al., 2006). For example, CD105$^{+}$ ASCs were more prone to differentiation into chondrocytes than CD105$^{-}$ ASCs (Ishimura et al., 2008; Jiang et al., 2010). CD105$^{+}$ MSCs were also more efficient in the infarcted heart (Gaebel et al., 2011), with stronger proliferative and colony formation abilities than CD105$^{-}$ MSCs (Lv et al., 2012). However, CD105$^{-}$ ASCs were more osteogenic (Jiang et al., 2010) and showed strong immunomodulation capacity (Anderson et al., 2013). Interestingly, five of the six USC$^{TA+}$ clones strongly expressed CD105, and four of the six USC$^{TA-}$ clones either did not express CD105 or were weakly positive. Compared to other CD markers, CD105$^{+}$ appeared to correlate with stemness. The correlation between CD105$^{+}$ USC and USC$^{TA+}$ of stem cells appears to have different capabilities. It is worth further investigation into the mechanism of correlation in both TA and CD105 marker cells with such different capabilities.

Telomerase and telomeres are strongly associated with cell renewal and proliferation, but it is controversial whether telomerase is associated with cell differentiation in MSCs (Zimmermann et al., 2003; Hiyama and Hiyama, 2007). The epigenetic nature of telomeres appears to depend on different human cell linages (Dogan and Forsyth, 2021). In adult human stem cells, both BMSCs and ASCs lack telomerase, but can retain their functional characteristics and multiple differentiation potential (Zimmermann et al., 2003; Hiyama and Hiyama, 2007). Interestingly, overexpression of telomerase resulted in telomere elongation, and TERT-transfected cells continued to proliferate and formed bone *in vivo* (Simonsen et al., 2002). However, mouse MSCs with their TA knocked down failed to differentiate into adipocytes or chondrocytes, even at early passage (Liu et al., 2004). Similarly, increased TA enhanced self-renewal ability, proliferation, and differentiation efficiency in TERT-overexpressing ES cells (Armstrong et al., 2005). High TA or the expression of TERT can therefore be regarded as a marker of undifferentiated ES cells. Downregulation of TA in differentiating EC cells was reported to be closely correlated with histone deacetylation and DNA methylation of the TERT gene (Lopatina et al., 2003). In this study, USCs$^{TA+}$ performed better in terms of multiple differentiation capacity in osteogenic, myogenic, and uroepithelial differentiation than USCs$^{TA-}$, indicating that telomerase is required for not only cell proliferation but also multiple differentiation in human USCs, which is a guarantee for future studies to determine whether USC$^{TA+}$ induce better *in vivo* tissue regeneration than USC$^{TA-}$.

**TABLE 6 |** Feature summary of human USCs with telomerase activity in $USC^{TA+}$ and $USC^{TA-}$ clones.

| | $USC^{TA+}$ | $USC^{TA-}$ |
|---|---|---|
| Cell renewal | | |
| - Population doubling | Higher | Lower |
| - Doubling time | Shorter | Longer |
| - Passages | More | More |
| Cell differentiation | | |
| - Osteogenic makers | Strong positive | Negative |
| - Smooth muscle makers | Strong positive | Weak expression |
| - Urothelial markers | Strong positive | negative |
| MSC markers | | |
| - CD44, CD73, CD90, CD146, | Strong positive | Strong positive |
| - CD25, CD31, CD34, CD45, CD117, CD140b HLA-DR/DP/DQ, HLA-G | Negative | Negative |
| - CD105 Strong positive | 4/6 | 2/6 |
| Age-dependent | | |
| - 20–40s | 73–77% of USC | 23–27% |
| - 50s | 59% of USC | 41% |
| **Safety** | | |
| - Chromosome stability | Yes | Yes |
| - Tumor Colone formation in aga | Negative | Negative |
| - Teratoma formation *in vivo* | Negative | Negative |

As telomerase is related to both normal stem cells and tumor stem cells, it is necessary to determine the alteration of karyotypes, with the *in vitro* agar assay and the *in vivo* risk of tumor formation of telomerase-positive cells for their safe transplantation application. Giemsa-based chromosomal banding and staining techniques are important for cytogenetics. USCs maintain a normal diploid chromosome recognized as 46 during long-term culture or overexpansion for up to p16 or 68 PD ($2^{68}$). Cytogenetic analyses showed USCs can safely expand *in vitro* (p4, p8, and p16) with no sign of immortalization or development of chromosomal abnormalities.

Cloning techniques with semisolid medium, such as agar gel for evaluating cell growth, are commonly used to study the biology of cancer cells due to their anchorage-independent growth requirements. The ability of cancer cells to proliferate without firm attachment (i.e., anchorage independence) is one of the best *in vitro* indicators of tumorigenicity. Importantly, $USCs^{TA+}$ do not present a propensity for spontaneous oncogenic transformation 60 days after *in vivo* implantation. The tumorigenic potential of $USCs^{TA+}$ was not found *in vitro* or *in vivo*. Thus, USCs as a new source of seed cells, which are non-invasive, highly proliferative, and abundant, can be used for tissue engineering and regenerative medicine.

TA appears to be related to the stemness of USCs (**Table 6**). $USCs^{TA+}$ survived for a significantly longer period with intact morphological appearance, rapid proliferation, and ample cell generation, and possessed more potent differentiation capacity than $USCs^{TA-}$. Both human $USCs^{TA+}$ and $USCs^{TA-}$ can be safely expanded *in vitro* maintaining normal karyotype and showed tumor-free formation after *in vivo* implantation, which makes them appropriate sources for cell-based therapies. Thus, TA could be an independent predictive factor for the regenerative capacity of USCs. In addition, human $USCs^{TA+}$ provides sufficient cell numbers for drug testing. TA is a good indicator of stemness (cell renewal and differentiation potential) of human adult stem cells, but a collection of primary cultured cells is required, which is cumbersome. Therefore, simpler and low-cost methods of measuring TA and telomeres in stem cells are highly desired.

## 5 CONCLUSION

This study demonstrated that human primary urinary stem cells with positive TA act as a distinct subpopulation with potential regeneration capacity in both cell proliferation and multiple differentiation. $USCs^{TA+}$ can more efficiently give rise to osteogenic, skeletal myogenic, smooth muscle, and urothelial cell lines than $USCs^{TA-}$. Importantly, despite that USCs display TA, they do not form teratoma, which provides a safe cell source for clinical application. In addition, the number of $USCs^{TA+}$ decline with increasing age. Future investigations should focus on understanding the role that physiological factors play in regulating both the temporal pattern of $USCs^{TA+}$ and their influence on the ability of these cells to participate in better tissue repair. Determining the requirements for the effect of TA on the paracrine effect of USC has important implications for understanding the anti-inflammatory, fibrosis inhibition, and redox effect of $USCs^{TA+}$. It will be beneficial to better understand alterations in this cell subpopulation throughout the human lifespan, and how they translate into, aging, renal dysfunction, drug-induced nephrotoxicity, or cancer, among others.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material,

further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the All human tissue samples were approved Human urine-derived stem cells (USC): All human tissue samples were approved for acquisition by the Wake Forest University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Experiments using nude mice were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

YZ supervision; YS and GL analyzed the data; YS, GL, RW, DM, XS, JM, XG, and AA contributed materials, reagents, analytic tools; YS, GL, and YZ wrote the original manuscript. All authors have read, edited, and approved the manuscript for publication.

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## REFERENCES

Anderson, P., Carrillo-Gálvez, A. B., García-Pérez, A., Cobo, M., and Martín, F. (2013). CD105 (Endoglin)-negative Murine Mesenchymal Stromal Cells Define a New Multipotent Subpopulation with Distinct Differentiation and Immunomodulatory Capacities. *PLoS One* 8, e76979. doi:10.1371/journal.pone.0076979

Armstrong, L., Saretzki, G., Peters, H., Wappler, I., Evans, J., Hole, N., et al. (2005). Overexpression of Telomerase Confers Growth Advantage, Stress Resistance, and Enhanced Differentiation of ESCs toward the Hematopoietic Lineage. *Stem Cells* 23, 516–529. doi:10.1634/stemcells.2004-0269

Bernardo, M. E., Zaffaroni, N., Novara, F., Cometa, A. M., Avanzini, M. A., Moretta, A., et al. (2007). Human Bone Marrow-Derived Mesenchymal Stem Cells Do Not Undergo Transformation after Long-Term *In Vitro* Culture and Do Not Exhibit Telomere Maintenance Mechanisms. *Cancer Res.* 67, 9142–9149. doi:10.1158/0008-5472.can-06-4690

Bharadwaj, S., Liu, G., Shi, Y., Markert, C., Andersson, K.-E., Atala, A., et al. (2011). Characterization of Urine-Derived Stem Cells Obtained from Upper Urinary Tract for Use in Cell-Based Urological Tissue Engineering. *Tissue Eng. Part A* 17, 2123–2132. doi:10.1089/ten.tea.2010.0637

Bharadwaj, S., Liu, G., Shi, Y., Wu, R., Yang, B., He, T., et al. (2013). Multipotential Differentiation of Human Urine-Derived Stem Cells: Potential for Therapeutic Applications in Urology. *Stem Cells* 31, 1840–1856. doi:10.1002/stem.1424

Bodin, A., Bharadwaj, S., Wu, S., Gatenholm, P., Atala, A., and Zhang, Y. (2010). Tissue-engineered Conduit Using Urine-Derived Stem Cells Seeded Bacterial Cellulose Polymer in Urinary Reconstruction and Diversion. *Biomaterials* 31, 8889–8901. doi:10.1016/j.biomaterials.2010.07.108

Cross, W. R., Eardley, I., Leese, H. J., and Southgate, J. (2005). A Biomimetic Tissue from Cultured Normal Human Urothelial Cells: Analysis of Physiological Function. *Am. J. Physiology-Renal Physiology* 289, F459–F468. doi:10.1152/ajprenal.00040.2005

Dogan, F., and Forsyth, N. R. (2021). Epigenetic Features in Regulation of Telomeres and Telomerase in Stem Cells. *Emerg. Top. Life Sci.* 5, 497–505. doi:10.1042/etls20200344

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S., et al. (2006). Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy Position Statement. *Cytotherapy* 8, 315–317. doi:10.1080/14653240600855905

Gaebel, R., Furlani, D., Sorg, H., Polchow, B., Frank, J., Bieback, K., et al. (2011). Cell Origin of Human Mesenchymal Stem Cells Determines a Different Healing Performance in Cardiac Regeneration. *PLoS One* 6, e15652. doi:10.1371/journal.pone.0015652

Ge, J., Cai, H., and Tan, W. S. (2011). Chromosomal Stability during *Ex Vivo* Expansion of UCB CD34+ Cells. *Cell Prolif.* 44, 550–557. doi:10.1111/j.1365-2184.2011.00779.x

Hiyama, E., and Hiyama, K. (2007). Telomere and Telomerase in Stem Cells. *Br. J. Cancer* 96, 1020–1024. doi:10.1038/sj.bjc.6603671

Hofmeister, L. H., Costa, L., Balikov, D. A., Crowder, S. W., Terekhov, A., Sung, H.-J., et al. (2015). Patterned Polymer Matrix Promotes Stemness and Cell-Cell Interaction of Adult Stem Cells. *J. Biol. Eng.* 9, 18. doi:10.1186/s13036-015-0016-x

Huang, Y., Liang, P., Liu, D., Huang, J., and Songyang, Z. (2014). Telomere Regulation in Pluripotent Stem Cells. *Protein Cell* 5, 194–202. doi:10.1007/s13238-014-0028-1

Ishimura, D., Yamamoto, N., Tajima, K., Ohno, A., Yamamoto, Y., Washimi, O., et al. (2008). Differentiation of Adipose-Derived Stromal Vascular Fraction Culture Cells into Chondrocytes Using the Method of Cell Sorting with a Mesenchymal Stem Cell Marker. *Tohoku J. Exp. Med.* 216, 149–156. doi:10.1620/tjem.216.149

Jiang, T., Liu, W., Lv, X., Sun, H., Zhang, L., Liu, Y., et al. (2010). Potent *In Vitro* Chondrogenesis of CD105 Enriched Human Adipose-Derived Stem Cells. *Biomaterials* 31, 3564–3571. doi:10.1016/j.biomaterials.2010.01.050

Kang, H. S., Choi, S. H., Kim, B. S., Choi, J. Y., Park, G.-B., Kwon, T. G., et al. (2015). Advanced Properties of Urine Derived Stem Cells Compared to Adipose Tissue Derived Stem Cells in Terms of Cell Proliferation, Immune Modulation and Multi Differentiation. *J. Korean Med. Sci.* 30, 1764–1776. doi:10.3346/jkms.2015.30.12.1764

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., et al. (1994). Specific Association of Human Telomerase Activity with Immortal Cells and Cancer. *Science* 266, 2011–2015. doi:10.1126/science.7605428

Kim, N., and Wu, F. (1997). Advances in Quantification and Characterization of Telomerase Activity by the Telomeric Repeat Amplification Protocol (TRAP). *Nucleic Acids Res.* 25, 2595–2597. doi:10.1093/nar/25.13.2595

Lafferty-Whyte, K., Cairney, C. J., Will, M. B., Serakinci, N., Daidone, M.-G., Zaffaroni, N., et al. (2009). A Gene Expression Signature Classifying Telomerase and ALT Immortalization Reveals an hTERT Regulatory Network and Suggests a Mesenchymal Stem Cell Origin for ALT. *Oncogene* 28, 3765–3774. doi:10.1038/onc.2009.238

Lang, R., Liu, G., Shi, Y., Bharadwaj, S., Leng, X., Zhou, X., et al. (2013). Self-renewal and Differentiation Capacity of Urine-Derived Stem Cells after Urine Preservation for 24 hours. *PLoS One* 8, e53980. doi:10.1371/journal.pone.0053980

Li, F., Ge, Y., Liu, D., and Songyang, Z. (2020). The Role of Telomere-Binding Modulators in Pluripotent Stem Cells. *Protein Cell* 11, 60–70. doi:10.1007/s13238-019-0651-y

Liu, L., Digirolamo, C. M., Navarro, P. A. A. S., Blasco, M. A., and Keefe, D. L. (2004). Telomerase Deficiency Impairs Differentiation of Mesenchymal Stem Cells. *Exp. Cell Res.* 294, 1–8. doi:10.1016/j.yexcr.2003.10.031

Lopatina, N. G., Poole, J. C., Saldanha, S. N., Hansen, N. J., Key, J. S., Pita, M. A., et al. (2003). Control Mechanisms in the Regulation of Telomerase Reverse Transcriptase Expression in Differentiating Human Teratocarcinoma Cells. *Biochem. Biophysical Res. Commun.* 306, 650–659. doi:10.1016/s0006-291x(03)01033-7

Lu, L. L., Liu, Y. J., Yang, S. G., Zhao, Q. J., Wang, X., Gong, W., et al. (2006). Isolation and Characterization of Human Umbilical Cord Mesenchymal Stem

Cells with Hematopoiesis-Supportive Function and Other Potentials. *haematologica* 91, 1017–1026.

Lv, X.-J., Zhou, G.-D., Liu, Y., Liu, X., Chen, J.-N., Luo, X.-S., et al. (2012). *In Vitro* proliferation and Differentiation of Adipose-Derived Stem Cells Isolated Using Anti-cd105 Magnetic Beads. *Int. J. Mol. Med.* 30, 826–834. doi:10.3892/ijmm.2012.1063

Nava, M. B., Catanuto, G., Pennati, A. E., Rocco, N., Spano, A., Villa, R., et al. (2015b). Lack of Activation of Telomere Maintenance Mechanisms in Human Adipose Stromal Cells Derived from Fatty Portion of Lipoaspirates. *Plast. Reconstr. Surg.* 135, 114e–123e. doi:10.1097/PRS.0000000000001008

Nava, M. B., Catanuto, G., Pennati, A. E., Rocco, N., Spano, A., Villa, R., et al. (2015a). Lack of Activation of Telomere Maintenance Mechanisms in Human Adipose Stromal Cells Derived from Fatty Portion of Lipoaspirates. *Plastic Reconstr. Surg.* 135, 114e–123e. doi:10.1097/prs.0000000000001008

Simonsen, J. L., Rosada, C., Serakinci, N., Justesen, J., Stenderup, K., Rattan, S. I. S., et al. (2002). Telomerase Expression Extends the Proliferative Life-Span and Maintains the Osteogenic Potential of Human Bone Marrow Stromal Cells. *Nat. Biotechnol.* 20, 592–596. doi:10.1038/nbt0602-592

Sun, J., Xing, F., Zou, M., Gong, M., Li, L., and Xiang, Z. (2021). Comparison of Chondrogenesis-Related Biological Behaviors between Human Urine-Derived Stem Cells and Human Bone Marrow Mesenchymal Stem Cells from the Same Individual. *Stem Cell Res. Ther.* 12, 366. doi:10.1186/s13287-021-02370-1

Thongon, N., Ma, F., Santoni, A., Marchesini, M., Fiorini, E., Rose, A., et al. (2021). Hematopoiesis under Telomere Attrition at the Single-Cell Resolution. *Nat. Commun.* 12, 6850. doi:10.1038/s41467-021-27206-7

Wang, F., Yin, Y., Ye, X., Liu, K., Zhu, H., Wang, L., et al. (2012). Molecular Insights into the Heterogeneity of Telomere Reprogramming in Induced Pluripotent Stem Cells. *Cell Res.* 22, 757–768. doi:10.1038/cr.2011.201

Wu, S., Wang, Z., Bharadwaj, S., Hodges, S. J., Atala, A., and Zhang, Y. (2011). Implantation of Autologous Urine Derived Stem Cells Expressing Vascular Endothelial Growth Factor for Potential Use in Genitourinary Reconstruction. *J. Urology* 186, 640–647. doi:10.1016/j.juro.2011.03.152

Zhang, Y., Mcneill, E., Tian, H., Soker, S., Andersson, K.-E., Yoo, J. J., et al. (2008). Urine Derived Cells Are a Potential Source for Urological Tissue Reconstruction. *J. Urology* 180, 2226–2233. doi:10.1016/j.juro.2008.07.023

Zimmermann, S., Voss, M., Kaiser, S., Kapp, U., Waller, C. F., and Martens, U. M. (2003). Lack of Telomerase Activity in Human Mesenchymal Stem Cells. *Leukemia* 17, 1146–1149. doi:10.1038/sj.leu.2402962

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REVIEW
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# Stem Cell Therapy in Limb Ischemia: State-of-Art, Perspective, and Possible Impacts of Endometrial-Derived Stem Cells

*Saeed Khodayari[1,2,3†], Hamid Khodayari[2,3,4†], Somayeh Ebrahimi-Barough[1], Mehdi Khanmohammadi[5], Md Shahidul Islam[1], Miko Vesovic[6], Arash Goodarzi[1], Habibollah Mahmoodzadeh[2], Karim Nayernia[3], Nasser Aghdami[4,7]* and Jafar Ai[1]**

[1]Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Tehran University of Medical Science, Tehran, Iran, [2]Breast Disease Research Center, Tehran University of Medical Sciences, Tehran, Iran, [3]International Center for Personalized Medicine (P7MEDICINE), Düsseldorf, Germany, [4]Department of Regenerative Medicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran, [5]Skull Base Research Center, The Five Senses Institute, School of Medicine, Iran University of Medical Sciences, Tehran, Iran, [6]Department of Mathematics, Statistics, and Computer Science, University of Illinois at Chicago, Chicago, IL, United States, [7]Department of Infectious Diseases and Tropical Medicines, Tehran University of Medical Sciences, Tehran, Iran

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**Edited by:**
Sudjit Luanpitpong,
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Zhihui Dong,
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Regina Coeli dos Santos Goldenberg,
Federal University of Rio de Janeiro, Brazil

***Correspondence:**
Jafar Ai
jafar_ai@tums.ac.ir
Nasser Aghdami
nasser.aghdami@royaninstitute.org

†These authors share senior authorship

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As an evidence-based performance, the rising incidence of various ischemic disorders has been observed across many nations. As a result, there is a growing need for the development of more effective regenerative approaches that could serve as main therapeutic strategies for the treatment of these diseases. From a cellular perspective, promoted complex inflammatory mechanisms, after inhibition of organ blood flow, can lead to cell death in all tissue types. In this case, using the stem cell technology provides a safe and regenerative approach for ischemic tissue revascularization and functional cell formation. Limb ischemia (LI) is one of the most frequent ischemic disease types and has been shown to have a promising regenerative response through stem cell therapy based on several clinical trials. Bone marrow-derived mononuclear cells (BM-MNCs), peripheral blood CD34-positive mononuclear cells ($CD34^+$ PB-MNCs), mesenchymal stem cells (MSCs), and endothelial stem/progenitor cells (ESPCs) are the main, well-examined stem cell types in these studies. Additionally, our investigations reveal that endometrial tissue can be considered a suitable candidate for isolating new safe, effective, and feasible multipotent stem cells for limb regeneration. In addition to other teams' results, our

**Abbreviations:** **ANGPT2,** angiopoietin-2; **BDNF,** brain-derived neurotrophic factor; **BM-MNCs,** bone marrow mononuclear cells; **CCL-2,** C-C motif chemokine ligand 2; **CCL5,** C-C motif chemokine ligand 5; **$CD34^+$ MNCs,** CD34-positive mononuclear cells; **C-Kit,** tyrosine protein kinase Kit; **CXCL1,** X-C motif chemokine ligand 1; **CXCL5,** X-C motif chemokine ligand 5; **CXCL8,** C-X-C motif chemokine ligand 8; **EGF,** epidermal growth factor; **ESCS,** endometrial stem cells; **ERC,** endometrial regenerative cells; **ESPCs,** endothelial stem/progenitor cells; **GATA2,** GATA-binding factor 2; **HLA-DR,** human leukocyte antigen—DR; **ANGPT2,** angiopoietin-2; **ICAM-1,** intercellular adhesion molecule 1; **IL-10,** interleukin-10; **KDR,** kinase insert domain receptor; **MSCs,** mesenchymal stem cells; **NMPB-ACPs,** non-mobilized peripheral blood angiogenic cell precursors; **NO,** nitric oxide; **NOS,** nitric oxide synthase; **IL-8,** interleukin-8; **PDGF,** platelet-derived growth factor; **PDGFB,** platelet-derived growth factor subunit B; **PGPC,** *Escherichia coli* K-12; **PROM1,** prominin 1; **SDF-1,** stromal cell-derived factor 1; **SGPC,** streptogrisin-C; **TGF-β,** transforming growth factor-β; **VCAM-1,** vascular cell adhesion protein 1; **VEGF,** vascular endothelial growth factor; **VEGFR,** vascular endothelial growth factor receptor. **GFAP,** glial fibrillary acidic protein; **SOX,** SRY (sex-determining region Y)-Box 1; **FOXO-3,** forkhead box O3; **TLX,** orphan nuclear receptor TLX; **IGF-1,** insulin-like growth factor-1; **GDNF,** glial cell-derived neurotrophic factor; **NGF,** nerve growth factor.

in-depth studies on endometrial-derived stem cells (EnSCs) have shown that these cells have translational potential for limb ischemia treatment. The EnSCs are able to generate diverse types of cells which are essential for limb reconstruction, including endothelial cells, smooth muscle cells, muscle cells, and even peripheral nervous system populations. Hence, the main object of this review is to present stem cell technology and evaluate its method of regeneration in ischemic limb tissue.

**Keywords: stem cell therapy, limb ischemia, state-of-art, perspective, molecular mechanism, endometrial-derived stem cells, regeneration**

## INTRODUCTION

Peripheral arterial disease (PAD) results in a wide range of organ dysfunction and organ failure in humans. It has been reported that over 200 million PAD cases had been diagnosed just in 2017 globally (Fowkes et al., 2017). Meanwhile, limb ischemia (LI) in particular leads to a large number of these types of disorders (Torbjörnsson et al., 2021). Among these, acute limb ischemia (ALI) and critical limb ischemia (CLI) are considered the most common diagnostic cases of LI, which occur when there is a sudden lack of circulation to limbs due to occlusion of a peripheral artery or bypass graft (Shoji et al., 2020; Torbjörnsson et al., 2021). Through limb ischemia, peripheral arterial disorders, atherosclerotic peripheral vascular disease, and embolic occlusion can directly arise by reducing the blood perfusion into the limb tissues (Narula et al., 2018). Pain in the ischemic regions, thickening of the toenails, skin infections, and limb ulcers are considered the main symptoms of LI (Lambert and Belch, 2013). Interventions such as vascular and endovascular surgery are commonly used as standard approaches to help regulate and promote circulation to ischemic limbs (Almasri et al., 2019). Despite prescribed treatments, a significant number of patients with LIs have to undergo a major lower limb amputation (Fowkes et al., 2017); hence, the development of a safe, minimally invasive, and effective strategy for regenerating degenerated tissues is regarded as the primary strategy for the treatment of the LI.

Nowadays, the undeniable advantage of using stem cell technology is widely acknowledged as a reliable, safe, and effective method for generating mature cells (Khodayari et al., 2021a; Khodayari et al., 2021b) and regenerating injured tissues and organs (Emadedin et al., 2012; Rigato et al., 2017; Khodayari et al., 2019; Hashemian et al., 2021). From a developmental and physiological view throughout the animal's lifetime, stem cells play a vital role in the hemostasis, healing, and regeneration of the organs (Ghazizadeh et al., 2018; Khodayari et al., 2019; Beeken et al., 2021). Based on diverse experimental and clinical studies, stem cell-based therapies have been deemed suitable for managing the damaged limb tissues and improving the LI's symptoms (Zafarghandi et al., 2010; Molavi et al., 2016; Gao et al., 2019).

So far, various types of multipotent stem/progenitor cells have been successfully tested as potential candidates for limb regeneration. The therapeutic potential of BM-MNCs, MSCs, $CD34^+$ MNCs, ESPCs, EnSCs, and neural stem cells (NSCs) has been tested on LI patients with different criteria (**Supplementary Table S1**). Our clinical trials have shown promising results in terms of safety, long-term advantages, and feasibility of the BM-MNC and MSC therapies for limb tissue regeneration/reconstruction targets (Zafarghandi et al., 2010; Emadedin et al., 2012; Molavi et al., 2016). Promoting neovascularization, myogenesis, and neurogenesis, as well as secretion of different paracrine/autocrine factors into the injury microenvironment (**Table 1**), would be the main therapeutic benefit for the damaged limbs resulting from these transplanted stem cells. In this case, inducing therapeutic angiogenesis into the ischemic regions is the chief regenerative mechanism that is promoted by the stem cells (Johnson et al., 2019). This therapeutic response is triggered through the stem cell differentiation to endothelial cells and secreting various types of pro-angiogenic factors into the injured tissue microenvironment (Khodayari et al., 2019). Based on experimental observations, the MSCs, EPCs, and EnSCs are the cells that show both the responses after transplantation (Shamosi et al., 2015; Bouland et al., 2021), while the MNCs have no significant differentiation potential, and it seems their efficacy is induced *via* regulating the ischemic tissue environment (Li et al., 2006). These facts raise the possibility that releasing pro-angiogenic factors can be the main factor for inducing therapeutic angiogenesis into the injured limb.

The EnSCs' biology, differentiation, and regeneration potential have been thoroughly studied during our investigative process. In this regard, we could track the neurogenic, angiogenic, and myogenic potential of the mouse and human EnSCs in both *in vitro* and *in vivo* conditions (Ai and Ebrahimi, 2010; Mobarakeh et al., 2012; Tabatabaei et al., 2013; Khademi et al., 2014; Tehrani et al., 2014; Shamosi et al., 2017). In addition to the basic and clinical observations we have summarized in **Table 1**, **Supplementary Table S1**, our studies could reliably conclude that the EnSCs may be a novel and useful source for regenerating various types of ischemic disorders, along with other stem/progenitor cells (Ai and Ebrahimi, 2010).

Although different clinical examinations report the benefits of cell therapy on the ischemic organs, there are multiple challenges and barriers to achieving satisfactory regeneration in practice. In 2019, Khodayari et al. (2019) hypothesized that the creation of a stressful inflammatory microenvironment following the acute phase of myocardial infarction can be a potential inherent factor in blocking the outcome of cell therapy in this ischemic region. We believe creating this type of natural restriction would be a destructive factor for stem cell therapy goals, not only for infarcted myocardium but also for all other types of ischemic

**TABLE 1 |** Characteristics, biological activity, and main clinical applications of the approved stem/progenitor cell types for treatment of limb ischemia.

| Stem cell type | Developmental origin | Sources | Phenotype | Regeneration potential/generated linages | Paracrine/ autocrine factors | Other clinical applications | References |
|---|---|---|---|---|---|---|---|
| BM-MNCs | Non-hematopoietic origins such as ectoderm, mesoderm, and endoderm | Bone marrow | $CD34^+$, PROM1 $(CD133)^+$, KIT $(C\text{-}Kit)^+$, $CD14^-$, and $CD45^-$ | Myogenesis, angiogenesis, osteogenesis, as well as hepatogenesis, and neural lineage differentiation | Cytokines and immune suppressors: NOS, IL-8, IL-10, and TGF-β Growth factors: EGF, PDGF, VEGF, and SDF-1 Chemokine/ surface markers: CXCL8, CXCL5, CXCL1, and CCL5 | Cardiovascular disorders | Heldman et al. (2014) |
| | | | | | | Diabetes and its related complications | (Dubsky et al., 2013; Wu et al., 2014) |
| | | | | | | Brain diseases and spinal cord injuries | (Sarasúa et al., 2011; Moniche et al., 2015; Liu et al., 2017) |
| | | | | | | Bone fracture and disease | Seebach et al. (2016) |
| | | | | | | Skin regeneration | Zhou et al. (2016) |
| | | | | | | Liver disease | Mohamadnejad et al. (2016) |
| MSCs | Somatic lateral plate mesoderm | Bone marrow, peripheral blood, umbilical cord blood/ tissue, as well as adipose, muscle, skin, and cardiac tissues | $CD73^+$, $CD90^+$, $105^+$, $CD34^-$, $CD45^-$, $D11b^-$, $CD14^-$, $CD19^-$, $CD79a^-$, and $HLA\text{-}DR^-$ | Myogenesis, angiogenesis, osteogenesis, ligament and tendogenesis as well as hepatogenesis, dipogenesis, and neural lineage differentiation | Cytokines/ immune suppressors: NO, IL-10, TGF-β, and CCL-2 Growth factors: EGF, PDGF, VEGF, BDNF, IGF-1 and SDF-1 Chemokines/ surface markers: galectin, ICAM-1, and VCAM-1 | Cardiovascular disorders | Kim et al. (2018) |
| | | | | | | Diabetes | Bhansali et al. (2017) |
| | | | | | | Brain and spinal cord injuries | (Xiao et al., 2018; Levy et al., 2019) |
| | | | | | | Multiple system atrophy | Singer et al. (2019) |
| | | | | | | Pulmonary and respiratory diseases | Wilson et al. (2015) |
| | | | | | | Hepatic disorders | Shi et al. (2012) |
| | | | | | | Bone fracture and disease | Emadedin et al. (2018) |
| | | | | | | Kidney disease | (Makhlough et al., 2017; Makhlough et al., 2018) |
| | | | | | | Autoimmune disease | Liang et al. (2018) |
| | | | | | | Skin regeneration | Kim et al. (2020) |
| PB-$CD34^+$ MNCs | Non-hematopoietic origins such as ectoderm, mesoderm, and endoderm | Peripheral blood, umbilical cord blood, and bone marrow | $CD34^+$, $CD14^-$, and $CD45^-$ | Myogenesis, angiogenesis, osteogenesis, as well as hepatogenesis, and neural lineage differentiation | Cytokines and immune suppressors: SGPC, PGPC, NOS, IL-8, IL-10, and TGF-β Growth factors: EGF, PDGF, VEGF, and SDF-1, and BDNF Chemokine/ surface markers: CXCL8, CXCL5, CXCL1, and CCL5 | Cardiovascular disorders | Tendera et al. (2009) |
| | | | | | | Hepatic disorders | Park et al. (2013) |
| | | | | | | Hematopoietic recovery flowing chemotherapy | Cancelas et al. (1998) |

(Continued on following page)

**TABLE 1 |** (*Continued*) Characteristics, biological activity, and main clinical applications of the approved stem/progenitor cell types for treatment of limb ischemia.

| Stem cell type | Developmental origin | Sources | Phenotype | Regeneration potential/generated linages | Paracrine/ autocrine factors | Other clinical applications | References |
|---|---|---|---|---|---|---|---|
| ESPCs/ NMPB-ACPs | Endothelial and hematopoietic cell lineages | Peripheral blood, umbilical cord blood/ tissue, hemogenic endothelium, as well as adipose, muscle, skin, and cardiac tissues | $CD34^+$, $CD133^+$, ANGPT2+, CD144+, $VEGFR^+$, $GATA2^+$, $PDGFB^+$, $CD31^+$, $CD14^-$, and $KDR^-$ | Neovascularizationa and re-endothelialization | Cytokines and immune suppressors: IL-10, eNOS, and TGF-β | Cardiovascular disorders | (Lee et al., 2015; Zhu et al., 2016) |
| | | | | | Growth factors: VEGF-A, VEGF-B, SDF-1, and IGF-1 | Osteonecrosis | Daltro et al. (2015) |
| | | | | | Chemokine and surface markers: CXCR-4 and VCAM-1 | Diabetes and its related complications | Tanaka et al. (2014) |
| CTX/ NSCs | Ectoderm | Brain and spinal cord SVZ, oncogene immortalized stem cells, neurospheres, and embryonic stem cell (ES)-derived neural cells | $CD184^+$, $CD24^+$, $nestin^+$, $FGF\text{-}R^+$, $GFAP^+$, $SOX1/2^+$, $FOXO\text{-}3^+$, $TLX^+$, $CD271^-$, and $CD44^-$ | Neural lineage differentiation | Growth factors: EGF, b-FGF, IGF-1, VEGF, GDNF, NGF, and BDNF | Brain and spinal cord injuries | (Kalladka et al., 2016; Curtis et al., 2018) |
| | | | | | | Brain cancer treatment | Portnow et al. (2017) |
| | | | | | | Peripheral arterial disease | |
| EnSCs/ ERCs | Mesoderm | Endometrium | $CD146^+$, $PDGF\text{-}R\beta^+$, $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$, $SSEA\text{-}1^-$, $CD34^-$, $CD31^-$, and $CD45^-$ | Myogenesis, angiogenesis, osteogenesis, ligament and tendogenesis as well as hepatogenesis, adipogenesis, and neural lineage differentiation | Cytokines/ immune suppressors: NO, IL-10, TGF-β, and CCL-2 | Cardiovascular disorders | Bockeria et al. (2013) |
| | | | | | Growth factors: EGF, PDGF, VEGF, BDNF, IGF-1, and SDF-1 | Peripheral vascular diseases | Zhong et al. (2009) |
| | | | | | Chemokines/ surface markers: galectin, ICAM-1, and VCAM-1 | | |

disorders. It seems that 1) developing modern technologies to reduce the cost of manufacturing stem cells at the highest level, 2) creating next-generation stem cells compatible with the ischemic microenvironment, and 3) presenting new available stem cell sources with greater regenerative potential will be the adequate targets to achieve more efficient tissue regeneration in practice. The scope of this review article is to first describe the molecular mechanisms of pathogenesis of limb ischemia. After summarizing our clinical experiences and the other latest clinical trials on LI stem cell therapy, we will present our group's obtained evidence that could illustrate the EnSCs' therapeutic impacts on limb regeneration. Finally, we will outline the limitations and perspectives of the cell therapy approach for the regeneration of limb ischemia.

## LIMB ISCHEMIA PATHOGENESIS: PATHWAYS AND MECHANISMS

Based on recent studies, peripheral arterial disorder is the main factor in the progression of hind limb ischemia (HLI). Chronic and acute arterial occlusions are caused primarily by occlusive arterial diseases and could block the blood perfusion into the limbs, as well as related tissues and organs (Heo et al., 2017). During the ischemia pathogenesis, decreasing the hypoxic cell's metabolism, increasing the level of intracellular free radicals, and forming a stressful inflammatory network in the ischemic tissue microenvironment can negatively disturb the cellular population's viability and function (Khodayari et al., 2019).

It is reported that oxidative stresses, which interfere by increasing intracellular levels of reactive oxygen and nitrogen species (ROS and RNS), are the primary disruptive factors in the ischemia pathogenesis path (Schröder et al., 2012; Bagheri et al., 2016). Meanwhile, all limb tissue cell types, including endothelial cells, skeletal myocytes, fibroblasts, and peripheral neurons, have been shown to present identical pathogenic responses to intracellular ROS and RNS generation (Steiling et al., 1999; Wang et al., 2015; Cheng et al., 2016). Activation of reactive species can directly cause cell damage through DNA sequence alteration, gene amplification, and expression of some proto-oncogenes and tumor suppressor genes (Bagheri et al., 2016; Qiu et al., 2019). In most cellular populations, mitochondria organelles are the main targets of ROS-mediated signaling in the path of HLI pathogenesis. In more detail, production of ROS

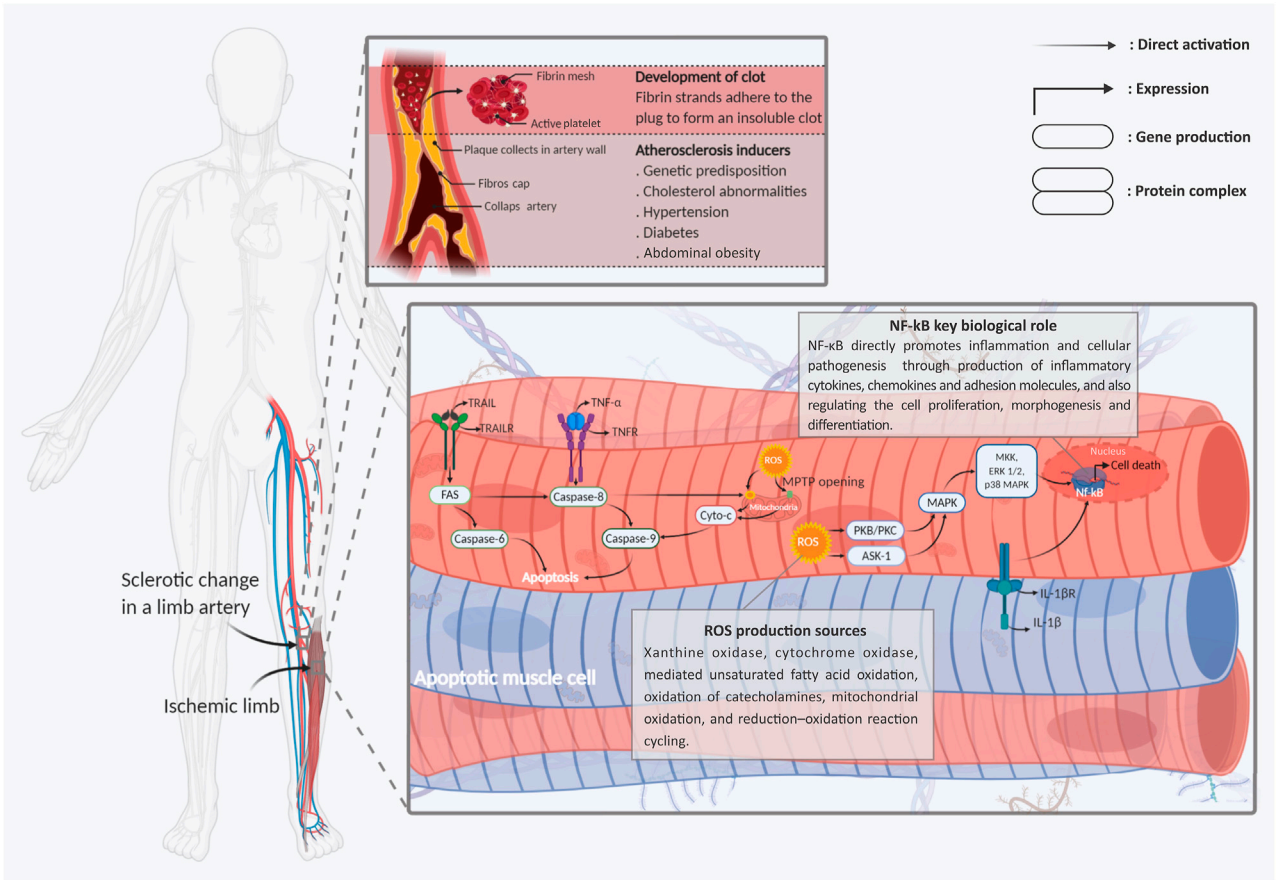


**FIGURE 1 |** Schematic representation of the main molecular mechanisms involved in limb ischemic pathogenesis. Two types of apoptotic skeletal muscle cells with fragmented nuclei under hypoxic stress and skeletal muscle cells are shown. Following ischemia, endogenic ROS production is induced early after injuries. Cytoplasmic ROS *via* chains of mPTPs opening of the mitochondrial membrane, releasing of the cyto c into the cells' cytoplasm, and activation and intra-nucleus accumulation of Nf-kB through MAPK signaling stimulation, leads to the muscle cells' apoptosis and necrosis. In addition to, stimulation of the TNF-R1/2, IL-1R, and TRAIL-R, as the main death ligands, by activation of the caspase cascades has a dominant role in developing the muscle cell's injuries. **Abbreviations**: Cyt-C, cytochrome-C; ERK, extracellular signal-regulated kinases; Fas, apoptosis antigen 1 (APO-1 or APT); IL-1R, interleukin-1 receptor; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor kappa-light-chain enhancer of activated B cells; ROS, reactive oxygen spices; TNF-R, tumor necrosis factor receptor; TNF-α, tumor necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TNF-related apoptosis-inducing receptor. Figure created with BioRender.com.

is directly controlled by the ischemic cell's mitochondria electron transport chain (ETC) overactivity (Indo et al., 2007). Moreover, ROS directly switches cytoplasmic $Ca^{2+}$ accumulation. This mechanism leads to interference of the injured cell's mitochondrial permeability transition pore (mPTP) opening and affects the mitochondrial membrane potential (Batandier et al., 2004). This mitochondrial mechanism immediately stimulates apoptosis cell death in the ischemic tissues by releasing cytochrome-c protein into the injured cell's cytoplasm, which, in turn, progresses the caspase cell death cascade (**Figure 1**). Aside from the cytochrome-c accumulation, some other harmful function cascades are caused by the ROS/mitochondia interaction with the injured cells. The cytoplasmic expression of apoptotic factors like B-cell lymphoma 2 (BCLl-2), BCL-2-associated X protein (BAX), BCL-2-associated death promoter (Bad), and glycogen synthase kinase 3β (GSK-3β) are the other main factors involved in this path (Perrelli et al., 2011) (**Figure 1**).

In contrast with the apoptotic mechanisms, several protective pathways can be initiated by the stressed mitochondria in response to ROS (Stein et al., 2017). The protein kinase C (PKC) signaling pathway is one of the main types of these protective mechanisms, which also plays multiple roles in the development and hemostasis of organs (Stein et al., 2017; Khodayari et al., 2019). In addition to the mitochondrial electron transport chain (ETC), the PKC and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, other main cell protective factors could be broadly released from different origins, including the xanthine oxidase, cyclooxygenase, and lipoxygenase (Leslie et al., 2004). In this regard, the generated ROS can directly target the PKC release into the injured cell cytoplasm through stimulation of the mitochondrial function. From another path, the activated PKC directly generates ROS production through NADPH phosphorylation (Lee et al., 2004). The activated NADPH improves cells' survival and function inside the stressful

environment through activation of protective signaling pathways, including the apoptosis signal-regulating kinase-1 (ASK1) and AKT pathways (Cosentino-Gomes et al., 2012) (**Figure 1**).

In addition to PKC, the mitogen-activated protein kinases (MAPKs), stress-activated protein kinases (SAPKs), phosphoinositide 3-kinase (PI3K), and ataxia-telangiectasia mutated (ATM) pathways can also serve as cell protective mechanisms which become activated as a result of ROS activity (Cosentino-Gomes et al., 2012). It has been identified that progression of both MAPK and SAPK activation of Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling can protect the injured cells from the disruptive impacts of oxidative stress. In this described cascade, activated PIK3 can significantly protect injured cells as well as distant cells by promoting 3 phosphoinositide-dependent protein kinase-1/Akt (PDK1/Akt) signaling (Cosentino-Gomes et al., 2012). The aforementioned pieces of evidence intensively prove this hypothesis, which states that ROS can directly act as a "double-edged sword" in the HLI pathogenesis process (**Figure 1**).

In addition to the described mitochondrial-associated mechanisms, non-mitochondrial ROS-mediated cell death plays a major role in limb tissue oxidative stress. In this process, generation and activation types of well-known inflammatory network elements including tumor necrosis factor-α (TNF-α) and its downstream targets have a central role in evolving the cellular death and limb tissue degeneration (Seekamp et al., 1993). It seems that this referred pro-inflammatory cytokine stimulates the same pathological responses for all organs and tissues, as well as the HLI (Khodayari et al., 2019). It has been shown that in following the limb tissue ischemia, the TNF-α production is directly initiated by overexpression of some adhesion molecules from ischemic tissue's vascular endothelial cells (Simon et al., 2018). According to contemporary observations, TNF-α is the initial and primary pro-inflammatory paracrine factor that directly switches the cytokine changes in the ischemic microenvironment (Heath et al., 2018). TNF-α, through the stimulation of leukocytes, initiates the inflammation process and recruitment of the inflammatory immune cells into the ischemic injured limb tissue. Subsequently, as a result of this mechanism, the expression of other types of pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8), occurs in infarcted tissues (Khodayari et al., 2019). It should be noted that this complex and stressful environment is considered the main reason for different inflammatory/degenerative disorders (**Figure 1**).

Furthermore, following the TNF-α associated pathogenicity, stimulation of the TNF-α receptors (TNF-Rs) as one of the main inflammatory cell death ligands can play the pivotal role in tissue degeneration (Sedger and McDermott, 2014). Promoting nuclear factor-kappa B (NF-κB) cascade, following TNF-R activation, can induce the cellular death process (apoptosis and necroptosis) into the injured cells (Kim et al., 2011). Like TNF-α, IL-1β-induced NF-κB activation is another pathogenic factor that occurs after the HLI process (Khodayari et al., 2019). The expressed NF-κB, through the activation of other pro-apoptotic factors including the inducible nitric oxide synthase (iNOS), BH3 interacting domain death agonist (Bid), and c-Jun N-terminal kinases/P38 (JNK/P38), can conduct the limb cells toward apoptosis (Cnop et al., 2005). Finally, it should be mentioned that all of the aforementioned adverse mechanisms are just a meager part of the pathogenic and progressive process that is activated in the ischemic limb tissue microenvironment.

## STEM CELL THERAPY APPROACHES FOR LIMB ISCHEMIA IN PRACTICE

In the past decade, stem cell-based therapy approaches have become extremely useful facilitators for the regulation of different types of human disorders, including acute and chronic limb ischemia (Zafarghandi et al., 2010; Mardanpour et al., 2019). From a translational and practical view, the performance of some multipotent stem/progenitor cells, including the BM-MNCs, $CD34^+$ MNCs, MSCs, and ESPCs, has been approved and deeply studied in different clinical trials (**Supplementary Table S1**). Furthermore, the therapeutic effects of EnSCs and NSCs are investigated in some published and ongoing clinical trials on limb lesions (**Supplementary Table S1**). Generally, the therapeutic benefits from these stem cells can be used in all phases of limb ischemia (Jaluvka et al., 2020). However, types of signs include patient survival rate lower than 6 months, history of malignancy, chronic renal disorders on dialysis therapy, or ALI cases with an advanced inflammatory reaction considers as the contraindication of the LI stem cell therapy (Jaluvka et al., 2020).

In this section, we will briefly review the biological function and regenerative outcomes of the aforementioned stem cells based on the latest clinical observations. Finally, at the end of this section, we will present the EnSCs as an "all-in-one" therapeutic approach for limb regeneration, based on our findings.

### Bone Marrow-Derived Mononuclear Cells

Generally, the BM-MNCs, as a heterogeneous cell population, are carefully characterized through the positive expression of CD34, CD45, CD133, and stromal precursor antigen-1 (STRO1) markers from bone marrow aspirated cells (Liang T. W. et al., 2016). It has been reported that the injected BM-MNCs, through the release of various anti-inflammatory cytokines and growth factors (**Table 1**), could regulate the ischemic region's pathogenic mechanisms. In addition, the vasculogenic, myogenic, and neurogenic differentiation potentials of these cells have been clearly recognized by numerous investigations (**Table 1**). According to these complementary features, the BM-MNCs are considered to be a major population in the target regeneration. However, some main drawbacks, such as the high possibility of contamination leading to a long time to culture and mainly decreased cell volume and regenerative capacity due to the increasing age of patients, can affect the use of the BM-MNCs for limb regeneration (Laurence et al., 2015).

So far, different studies have been conducted in order to evaluate the benefits of BM-MNC transplantation as a means

of managing limb ischemic injuries (**Supplementary Table S1**). From 2008 to 2011, we launched two separate clinical trials (phases I/II randomized control trials) for the autologous BM-MNC therapy of patients with CLI (ClinicalTrials.gov identifiers: NCT00677404 and NCT01480414). In those continued clinical observations, we focused on the most pressing questions, including 1) is autologous transplantation of the BM-MNCs safe in patients with CLI, 2) how much simultaneous injection of granulocyte-colony stimulation factor (G-CSF) with the transplantation of BM-MNCs would increase the efficacy of cell therapy in CLI patients, 3) can interval implantation of the BM-MNCs improve the treatment efficacy, and 4) can the BM-MNCs interval injection increase collateral vessel formation in patients with limb ischemia? Following these trials, we stated that the administration of BM-MNCs is a safe and feasible approach for the treatment of patients. Our evaluations additionally showed that the intervention hand was able to improve some patients' clinical indexes, including pain-free walking distance and Wagner stage, besides reduction in ulcer size (Zafarghandi et al., 2010; Molavi et al., 2016).

In a successful study, Tateishi-Yuyama et al. (2002) published an article titled "therapeutic angiogenesis for patients with limb ischemia by autologous transplantation of bone-marrow cells." Their clinical trial has shown that direct intramuscular injection of $CD34^+$ BM-MNCs not only is able to induce an angiogenic response but can also significantly improve the patient's limb function and ankle-brachial pressure index (ABI) 6 months after cell therapy (Pignon et al., 2017). Similar to the aforementioned study, current clinical observations are also displaying an identical therapeutic impact that occurred from the BM-MNCs administration on the limb ischemia cases. Pignon et al. (2017), through intramuscular injection of the $CD34^+$ BM-MNCs on patients with CMI, observed a suitable and remarkable therapeutic response within 6 months after cell therapy. Finally, they concluded that "BMSC therapy reduced the risk of major amputations in patients presenting with nonrevascularizable CLI" (Pignon et al., 2017).

Similarly, autologous bone marrow mononuclear cell (ABMNC) therapy is another approach that improves measures of limb perfusion, rest pain, wound healing, and amputation-free survival at 1 year in patients with CLI. As an example, Tateishi-Yuyama et al., in a retrospective study, evaluated the incidence of cardiac, malignant, and other medical events relevant to the safety of cell therapy. They found that none of the patients developed tumorigenesis or clinically significant retinopathy. Mainly, they found that in arms treated with ABMNC, the rates of amputation-free survival, major amputation, and major adverse limb events were significantly higher than in the control group (Tateishi-Yuyama et al., 2002).

Although in recent years, scientists have announced some remarkable therapeutic methods for regenerating the limb ischemia cases based on the administration of BM-MNCs, various gaps and problems have already existed in this path (Liang T. W. et al., 2016). It seems that there was a correlation between the reputation and numbers of the implanted BM-MNCs and regeneration outputs in the IL cases. Hence, our observation in 2016 has been compared with the therapeutic output that resulted from a single and repeated (4 step) intramuscular injection of $1–2\times10^5$ BMDC/kg on CLI patients (**Figure 2**). The results of this study revealed a significant improvement in the repeated treated cases "ABI, visual analog scale, pain-free walking distance, and Wagner stage as well as a reduction in ulcer size" in comparison with the single-injected CLI patients (Zafarghandi et al., 2010). However, it should be noted that the BM-MNCs are also showing satisfactory efficacy in ischemic limb regeneration (**Supplementary Table S1**). The achievement, compared to the other heterogeneous and high-potent stem cell populations with a suitable regenerative potential, can be presented as an effective approach along the lines of managing limb ischemia regeneration.

## Mesenchymal Stem Cells

Based on several hypotheses, presenting a large level of MSCs into the BM-MNC population introduces a key method in order to manage an effective therapeutic strategy for the regeneration of ischemic disorders (Wang et al., 2018; Khodayari et al., 2019; Mardanpour et al., 2019). Moreover, among the BM-MNC cellular population, the MSCs have shown more effectiveness for improving the ischemic limb tissues based on the particular observations in comparison with the other stem cells (Mafi et al., 2011; Gupta et al., 2013). In addition to the bone marrow, the MSCs can be isolated from different origins like fatty pads, synovium, and cord blood (Khodayari et al., 2019). Secreting a high level of paracrine factors, increased proliferation and differentiation potential, ability to utilize as an allogeneic source, elevated resistance to inflammation as well as lower teratogenic/carcinogenic potential considers the main advantages of the MSCs for the regenerating targets (Khodayari et al., 2019; Khalighfard et al., 2021). In contrast, the requirement to use invasive methods for getting a biopsy sample, the dependence of the cells' proliferative and differentiation capacity with age, and the need to use various markers for producing a homogeneous population are the cells' main disadvantages (Matsiko et al., 2013). Phenotypically, in humans, the BMSCs are a fibroblast-like cell population that is generally distinguished by the positive expression of STOR1, CD73, CD90, and CD105, besides the negative expression of CD11b, CD14, and CD34. Also, as negatively marked, CD79a and HLA-DR can be noticed in the MSCs (Khodayari et al., 2019). In addition to the bone marrow, human MSCs can be isolated from different niches, including the adipose tissue (Yañez et al., 2006), umbilical cord blood (Hong et al., 2005), and dental tissue (Xu et al., 2020).

There are several successful clinical methods for evaluating the regenerative impacts of MSCs on limb ischemia disorders. Gupta et al. (2013) have shown that intramuscularly injected BM-MSCs not only were safe but also decreased the CLI patient's limb pain and improved their ABI and ankle brachial pressure index (ABPI). Also, they have shown that the numbers of the administrated CM-MSCs are able to positively change the cell therapy output in CLI cases (Gupta et al., 2013). Das et al. (2013), *via* an intra-arterial allogeneic MSC infusion into CLI patients, could recognize a safe and effective response in their study. Based on their reports, not only did the MSCs treated group show

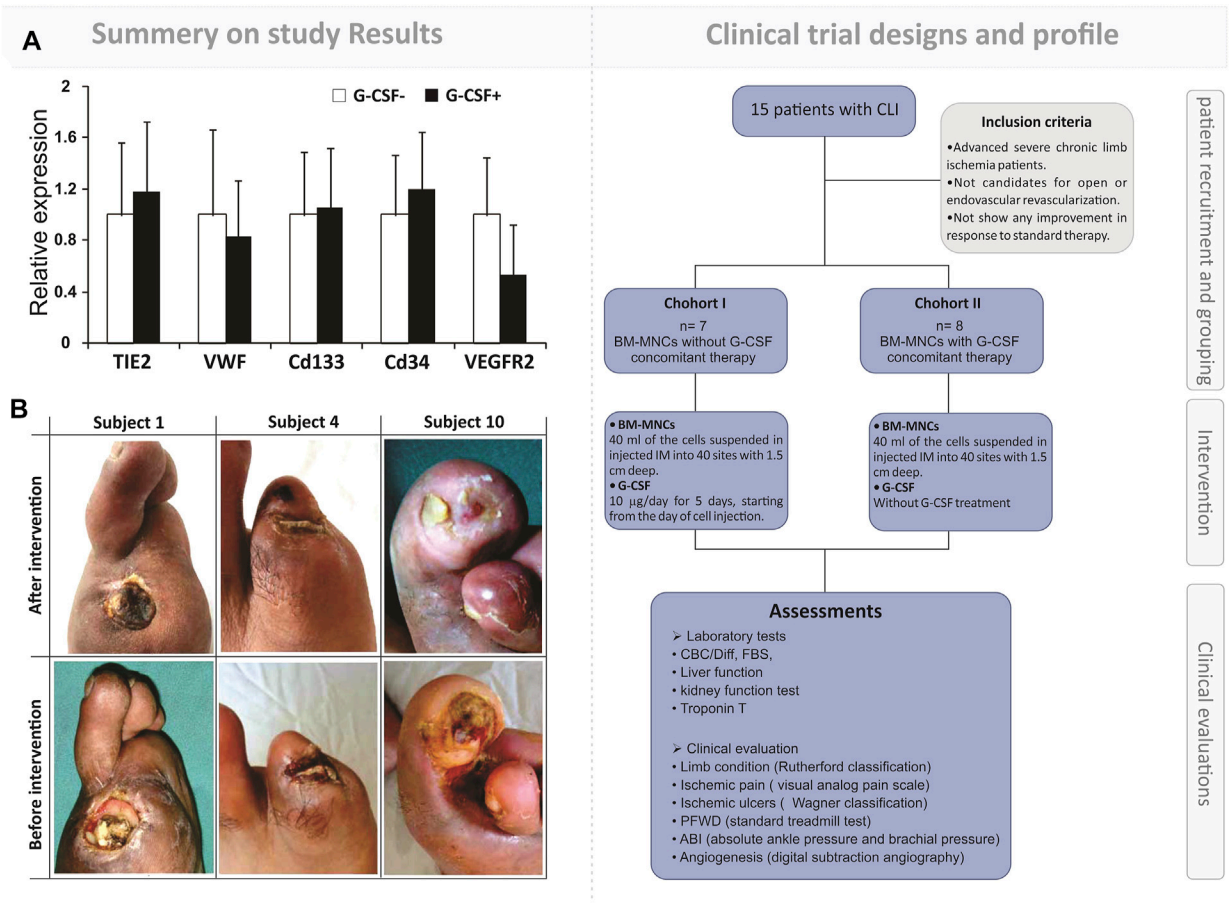


**FIGURE 2 |** Outcome of simultaneous autologous BM-MNC and G-CSF administration to patients with limb ischemia. **First part**: a brief representation of the flowchart for the clinical trial design and assessments. The study was a phase I randomized controlled trial on fifteen patients with severe CLI. The intervention's safety and efficacy were evaluated by laboratory analysis, clinical evaluation, and imaging. **Second part**: **(A)** qRT-PCR analysis of the expression of angiogenesis markers in two arms, BM-MNC alone vs. BM-MNC plus G-CSF. **(B)** Improvement in limb salvage after autologous BM-MNC therapy. Zafarghandi, Mohammad Reza, et al. Cytotherapy 12.6 (2010): 783–791.

significant pain relief, but it also greatly improved the other clinical indexes, including the visual analog scale (VAS), ABPI, and transcutaneous oxygen pressure ($TcPO_2$) (Das et al., 2013). Other clinical observations have only shown an unstable and limited regeneration response from the MSC subtype implant. Adipose-derived stromal/stem cells (ADSCs) are introduced as a mesenchymal stem-like population with the same molecular phenotype as MSCs (Zannettino et al., 2008). Moreover, Bura et al. (2014), through their phase I clinical trial on nonrevascularizable CLI patients, observed that even though intramuscularly injected ADSCs in the ischemic tissue were feasible and safe, the case's clinical index was not sufficiently improved after the cell therapy (Bura et al., 2014). This variation and huge difference between the MSCs and the MSC-like regenerative responses may be dependent on the variations of the cell's niches, paracrine/autocrine activity, and differentiation potential.

Generally, stimulating the angiogenic mechanisms in the MSCs seems to be a powerful approach for improving the neovascularization response from the implanted MSCs (Yu and Dardik, 2018; Goodarzi et al., 2020; Khalighfard et al., 2021). The upregulation of hypoxia-inducible transcription factor-1α (HIF-1α), as a chief angiogenic regulator, could be used as a feasible and effective procedure that could achieve this result. During this process, as the main downstream target of HIF-1α, activation of remarkable angiogenic signaling pathways including the vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and small mothers against decapentaplegic 3/4 (SMAD 3/4) can greatly improve the human MSCs' endothelial differentiation in the ischemic limb microenvironment (Huang et al., 2016). This hypothesis has been successfully proven by Wang et al., 2015), following their preclinical observations (Huang et al., 2016). Through intramuscular injection of $1\times10^6$ HIF-1α upregulated human MSCs, they recorded a meaningful angiogenic response and observed the injured limb tissue regenerate early after the cell transplantation (Howangyin et al., 2014).

## CD34-Positive Mononuclear Cells

As a population of MNCs, the $CD34^+$ mononuclear cells present a non-hematopoietic stem cell with an innate potential for tissue regeneration (Johnson et al., 2020). Different origins such as umbilical cord blood, bone marrow, and peripheral blood are utilized for the isolation of high-potent $CD34^+$ MNCs to be used as a therapeutic factor (Bender et al., 1994; Johnson et al., 2020). In this case, BM and PB-$CD34^+$ MNCs are the most commonly trialed cells for the treatment of ischemic limbs in practice (**Supplementary Table S1**). Generally, these cells can be isolated by positive expression of CD34 markers along with negative expression of CD14 and CD45 transmembrane proteins (**Table 1**). Different experimental observations have concluded that the PB-$CD34^+$ MNCs could directly generate types of cellular lineages like myocytes (Avitabile et al., 2011), endothelial cells (Krenning et al., 2009), and neural cell lineages (Reali et al., 2006) that are vital for injured limb regeneration. In addition to their differentiation potential, the release of different therapeutic factors, including 1) cytokines and immune suppressors, 2) growth factors and morphogens, 3) chemokines and surface regulatory markers, and 4) extracellular vesicles, can be used as other therapeutic effects resulting from the PB-$CD34^+$ MNCs implantation. A list of the main PB-$CD34^+$ MNCs secretomes is categorized in **Table 1**.

In order to increase the mobilization of the $CD34^+$ MNCs into circulation, the most common approach is to administer G-CSF days before cell isolation (Kawamoto et al., 2009). It has been shown that the pharmacological effects of G-CSF on BM stem cell populations are directly promoted *via* activation of signal transducer and activator of transcription 3 (STAT3) and rat sarcoma (RAS)/rapidly accelerated fibrosarcoma (RAF) signaling (Kamezaki et al., 2005). These pathways serve as types of central mechanisms which promote cellular proliferation and migration from bone marrow to transmission. So far, different experimental and clinical trials have evaluated the safety and effectiveness of the PB-$CD34^+$ MNCs in the various types of LIs (Kawamoto et al., 2009). Wahid et al. (2018) wrote in their meta-analysis for evaluating the "efficacy and safety of autologous cell-based therapy in patients with no-option critical limb ischemia" that the MNCs, mainly bone marrow-driven cells, are safe stem cells with more therapeutic potential for the regeneration of CLI (Wahid et al., 2018). In one of the earliest clinical trials, Kinoshita et al. (2012) examined the long-term clinical outcome of IM G-CSF-mobilized CD34 MNC injection in patients with CLI (Kinoshita et al., 2012). Their observation demonstrated that the IM injection of PB-$CD34^+$ MNCs had favorable and long-term clinical benefits in these types of patients. As a result, they have recorded an improvement in brachial pressure index and transcutaneous partial oxygen pressure up to 6 months after cell therapy (Kinoshita et al., 2012).

It seems that in addition to using a sufficient number of a viable and homogenous population of $CD34^+$ MNCs, the characteristics of subjects can be a determining issue for performing efficient cell therapy in LI patients. Pan et al. (2019), phase I/II clinical trial, showed that the total number of transplanted cells, patient's age, blood fibrinogen, arterial occlusion level, and TcPO2 directly affected the outcome of PB-$CD34^+$ MNCs therapeutic angiogenesis for no-option CLI cases (Pan et al., 2019). Moreover, the different observations identified suitable outcomes of the PB-$CD34^+$ MNC therapy in CLI patients with atherosclerotic peripheral arterial disease and Buerger's disease (**Supplementary Table S1**).

## Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) have been utilized to generate various cell types that have a key role in endothelial lining regeneration and neovascularization (Esquiva et al., 2018). Generally, EPCs are generated in the bone marrow environment by the $CD34^+/CD45^+/CD133^+$ hematopoietic stem cells (HSCs) and non-hematopoietic bone marrow cells, including the MSCs (Urbich and Dimmeler, 2004). EPCs are collected within the positive expression of CD133, CD34, and vascular endothelial growth factor receptor-2 (VEGFR2) directly from the bone marrow or circulating blood cells (Urbich and Dimmeler, 2004; Friedrich et al., 2006). It has been reported that in addition to neovascularization into the injured tissues, utilized EPCs can modulate the stressful organ's microenvironment through expression of diverse types of angiogenic growth factors like VEGF, hepatocyte growth factor (HGF), and insulin-like growth factor 1 (IGF-1) (Abe et al., 2013).

In the past, some clinical observations have considered the use of EPC's regenerative potential for patients with limb ischemia. As a primer clinical observation, Kudo et al. (2003) stated that intramuscular injection of the autologous $CD34^+$ EPC can be a powerful method for regenerating ischemic limb tissues. The results they presented after EPC therapy read "transcutaneous oxygen pressure in the foot increased and clinical symptoms improved." Newly visible collateral blood vessels were directly documented by angiography (Kudo et al., 2003). The important physiological function of the EPCs on the limb ischemia patient's therapeutic response rate has already been discovered. Based on this hypothesis, the lower neovascularization rate into the limb ischemia patients who are showing a minor therapeutic response to the treatments may be related to their EPC defect. Flow cytometry and EPC quantitative molecular analysis on ischemic limbs patients with a moderate risk of neovascularization, showed that not only the number of their circulating EPCs was lower but also the proliferation and differentiation potential of their collective EPCs was not as high as it was in the control cases (Yamamoto et al., 2004).

It has been found that stimulation of the EPCs with pro-inflammatory cytokines, like IL-1β, IL-3, and TNF-α, significantly decreases the proliferation and angiogenic potential of the cell. Accordingly, to perform an effective cell therapy on the ischemic limbs, based on using the EPCs, production of them at a large level are required (Peplow, 2014). We believe that this is the main disadvantage that leads to the reduction of this regenerative method's cost-effectiveness.

## Neural Stem Cells

Peripheral neuropathy associated with limb ischemia is one of the prevalent pathogenesis that is commonly observed in patients with vascular disease (Uccioli et al., 2018). "Chronic pain, foot

ulcers, foot infections, and amputations" manifest as chief complications caused by peripheral neuropathy (Hicks and Selvin, 2019). Nowadays, using stem cell technology for regeneration of the damaged peripheral neural system (PNS) presents an applicable, safe, and efficient method (Sullivan et al., 2016). This discovery of NSCs was an impactful development for the sake of eventually achieving a regenerative method for the injured PNS. The NSCs are a population of undifferentiated cells that naturally persist in the mammalian ventricular-subventricular zone (V-SVZ) and the subgranular zone (SGZ) (Fuentealba et al., 2012). In humans, this cellular population is noted by positive expression of CD184, CD24, nestin, fibroblast growth factor receptor (FGF-R), glial fibrillary acidic protein (GFAP), SRY-box transcription factors 1/2 (SOX1/2), forkhead box O3 (FOXO-3), and orphan Nuclear Receptor TLX, as well as negatively expressed CD271 and CD44 markers (**Table 1**). Different observations proved the neural linage differentiation potential of NSCs in both *in vitro* and *in vivo* conditions (Harbom et al., 2018). In addition to the release of various paracrine and growth factors, these stem cells can also express types of chemokines and cell migration receptors (**Table 1**).

The large-scale production of NSCs for managing an effective cell therapy in patients with PNS disorders presents a principal challenge. Extreme risks of taking a biopsy from a patient's SVZ and SGZ, the presence of small numbers of NSCs in the received tissue, and finally, the poor proliferative potential of the hNSCs primary cultures are some of the limitations presented in this approach (Kaneko et al., 2011). However, like the EPCs, resistance to the ischemic organ inflammatory responses along with the high cost of the NSCs' isolation, culture, and purification prevents the widespread use of these cells in the practice (Pluchino et al., 2020). Nowadays, using gene transfection technology to generate off-the-shelf NSCs lines serves as a feasible and satisfactory approach. As a pioneer product, the human NSC-line CTX (CTX0E03) created with the MYCL proto-oncogene (c-myc)-ERTAM transfection performs as an off-the-shelf allogeneic stem cell for the CNS and PNS regeneration targets, in practice (Yoon et al., 2020). Results of the Muir et al. (2020) clinical trial on twenty-three patients with stroke confirmed that intracerebral implantation of the CTX cells was not only a feasible approach for nerve regeneration but also had a positive outcome on the subject's motor recovery up to 12 months after implantation (Muir et al., 2020). A phase I, randomized, controlled trial has been launched in order to investigate the safety of CTX0E03 cell line implantation in patients with "insulinized diabetic patients type 2 with CLI in the lower limb" (ClinicalTrials.gov Identifier: NCT02287974). Although results have yet to be posted from this ongoing study, evidence raised from Muir et al. (2020) study may suggest that using these universal cell lines could be a safe and efficient cell therapy method for patients with LI.

## ENDOMETRIAL-DERIVED STEM CELLS: ALL-IN-ONE FOR LIMB REGENERATION

As we explained, different stem cells have been utilized in many preclinical and clinical experiments over the past 5 decades due to their extraordinary capacity for differentiation into multiple lineages, immunomodulation, and angiogenic properties (**Supplementary Table S1**). During the past few years, our team's extensive observations into EnSCs have displayed this stem cell's potential for regenerating ischemic organs as well as limb tissue. The presence of stem cells in the endometrium was first speculated upon by Prianishnikov (1978) (Prianishnikov, 1978). Chan et al. (2004) also confirmed the presence of clonogenic stem cell populations in the endometrium tissue (Chan et al., 2004). We already proved that this mentioned regenerative capacity in the endometrium tissue is due to the presence of EnSCs that were demonstrated to be immuno-privileged in comparison with other cell types (Shamosi et al., 2015), shedding new light on cell-based therapies and rendering these cells a promising resource in limb tissue regeneration (**Figure 3**). The human endometrium is a highly regenerative tissue due to the fact that a complete regeneration of the uterus can be achieved after almost total resection of the endometrium, with the renovated endometrium still maintaining its ability to support gestation (Murphy et al., 2008). Monthly cyclical endometrial growth, differentiation, and regression are governed by a fine-tuned interplay between ovarian sex steroid hormones and numerous cell types. It has been shown that estrogen replacement therapy can regenerate atrophied endometrium (Evans et al., 2016). The endometrial tissue is composed of epithelial stem cells (0.22%), mesenchymal stem cells (1.25%), and side population stem cells (Sasson and Taylor, 2008). During menstruation, all of these cells could be found as menstrual stem cells or endometrial regenerative cells (ERC) (Verdi et al., 2014). Like the other sources, endometrial MSCs have recently emerged as a promising alternative source of therapeutic MSCs due to their unique ability to undergo smooth muscle differentiation (Rink et al., 2017), angiogenic (Cabezas et al., 2018), adipogenic (Ye and Yuan, 2007) and robust immunomodulation properties (Cortés-Araya et al., 2018). *In vitro* analysis reported that the EnSCs positively express stage-specific embryonic antigen-4) SSEA-4 (and octamer-binding transcription factor 4 (OCT4) markers and decrease OCT4 expression with increasing passages that confirm its stemness (Piccinato et al., 2015). The EnSCs also expressed high levels of CD73, CD90, CD105, and CD166, but they lacked CD14, CD34, and CD45 expression, which are very similar to the minimum characteristics of MSCs defined by the committee of the International Society for Cellular Therapy (Piccirillo et al., 2013). The EnSCs also have a high proliferation, multidirectional, and differentiation capacity that is indicative of the potential for tissue regeneration (Ai and Ebrahimi, 2010).

As an advantage, the EnSCs have been shown to have the same immunophenotype as the MSCs. These cells express MHC class I (HLA-ABC) but not MHC II (HLA-DR). They have the capability to modulate immune system functions, reduce proinflammatory cytokines including the TNF-α and interferon gamma (IFN-γ), and have been shown to inhibit mixed lymphocyte reactions (MLR) in different experiments (Cheng et al., 2017). As a result, EnSCs have been shown to have low immunogenicity and various *in vivo* experiments

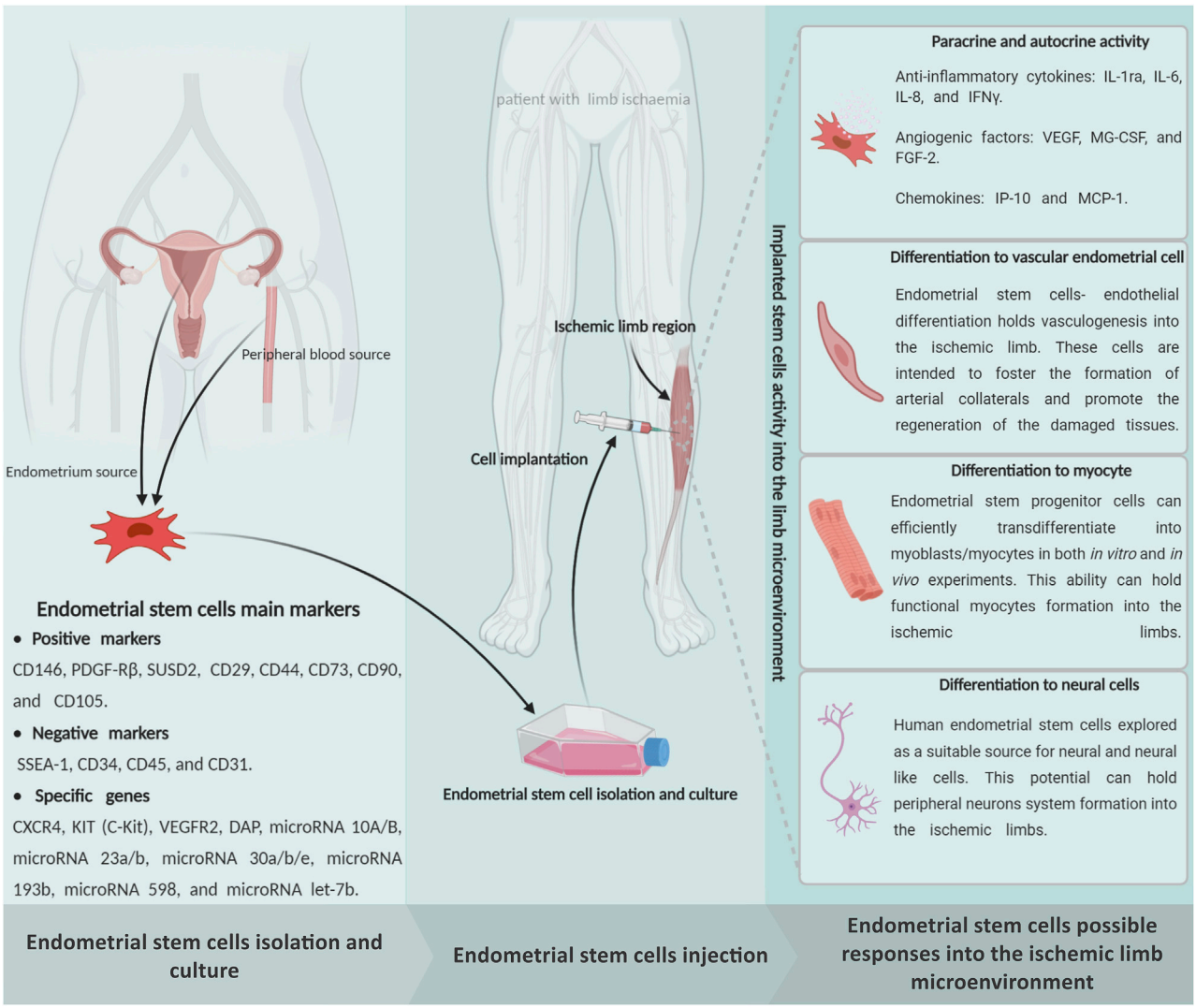


**FIGURE 3 |** Schematic representation of the endometrial stem cells possible impact on the limb ischemia regeneration subjects. The endometrial stem cells can be directly isolated from an endometrium biopsy through exposure of endometrial, mesenchymal, and endothelial specific markers. It seems that implantation of the *in vitro* expanded endometrial stem cells into the ischemic regions is able to protect the damaged cells from cellular death through a powerful paracrine/autocrine activity. In addition to, as a multipotent stem cell, the endometrial stem cells can generate the main limb linage cells, which may promote effective regeneration in the ischemic limbs. Figure created with BioRender.com.

have been performed without the occurrence of immune rejection (Giudice, 1994). Moreover, as well as the MSCs, the EnSCs can directly synthesize and discharge several types of paracrine factors, including the TGFα, EGF, and IGF-I (**Figure 3**). IGF-I, TGFα, epidermal growth factor receptor (EGFR), basic fibroblast growth factor (bFGF), and platelet-derived growth factor-β (PDGFβ) receptor, which are expressed in epithelial cells, are upregulated during expeditious growth in the proliferative stage of stem cells. Leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), and stem cell factor (SCF) have an essential contribution to endometrial regeneration (Chegini et al., 1992). All of the EnSC paracrine factors have an essential role in ischemic limb regeneration.

The novel hypothesis considers angiogenesis as the most remarkable regenerative approach, along with stem cell therapy for limb ischemia patients. We strongly believe, due to the high similarity between the EnSCs and the MSCs, that evaluating the MSCs' angiogenic responses in the ischemic tissue's microenvironment can be an accurate model to study for the EnSCs' angiogenic behaviors (Ai and Ebrahimi, 2010). The MSCs exhibit angiogenic properties by not only paracrine signaling but also by regulating this response through direct cellular involvement. An array of angiogenic factors secreted by MSCs, for example, VEGF, SDF1, FGF2, HGF, angiopoietin-1 (ANG1), and monocyte chemoattractant protein-1 (MCP-1), act as key growth factors for primary vessel formation and its stabilization (Tao et al., 2016).

Moreover, *in vitro* and *in vivo* studies showed that the microvesicles (>200 μm) and exosomes (~50–200 μm) secreted by MSCs transport proangiogenic growth factors and miRNA (Phinney and Pittenger, 2017). A proteomic analysis showed that enhanced therapeutic angiogenesis, resulting from the secretion of MSC-derived exosomal proteins (PDGF, FGF, EGF, NF-κB pathway-affiliated proteins, etc.) into the ischemic region, has been observed after MSC therapy (Anderson et al., 2016). X.Liang T. W. et al., 2016) demonstrated that adipose MSC-derived exosomes transfer miR-125a to endothelial cells and promote angiogenesis (Liang X. et al., 2016). Also, Gong et al. (2017) have investigated whether the proangiogenic exosomal miRNA (miR-424, miR-30c, miR-30b, let-7f, etc.), derived from MSCs, can be transferred into endothelial cells (Gong et al., 2017).

Many investigations have shown that BM-MSCs have a strong capability for angiogenesis induction (Kinnaird et al., 2004). Clearly, the EMSCs and BM-MSCs share some common cell surface markers like CD90 and CD105, while lacking CD45 and CD34 (Keating, 2012). A series of experiments have been performed *in vitro* and *in vivo* to assess the proof of an angiogenic concept on endometrial derived stem cells, and they showed that EnSCs could stimulate angiogenesis (Murphy et al., 2008). Results from our team's studies have shown that human EnSCs could proliferate and sprout new blood vessels in 3D fibrin matrix supported culture (Esfandiari et al., 2007a; Esfandiari et al., 2007b; Esfandiari et al., 2008). Similarly, in 2013, we were able to prove the EnSCs' direct potential to generate $CD34^+$ endothelial cells (Ai et al., 2013). In addition, according to data from one of our *in vitro* experiments, human EnSCs have shown that they can be differentiated into endothelial-like cells in the presence of FGF-2 and VEGF on a nanofibrous scaffold (Shamosi et al., 2017). Following this discovery, in 2004, we demonstrated the vascular network formation and the angiogenic potential of EnSCs through an *in vitro* examination (Esfandiari et al., 2008). It is important to note that EnSCs are found to be clonogenic mesenchymal-like cells (Gargett et al., 2009), which express pericyte markers and are localized in the perivascular space of endometrial small vessels (Berger et al., 2005). These cells play a central role in the formation of endometrial stromal vascular tissue and vascularization through the secretion of pro-angiogenic and growth-supporting factors. Canosa et al., 2017) concluded that the EnSCs could support the endothelial cell's ability to differentiate by taking information from the endothelium and new blood vessels (Canosa et al., 2017). Due to these findings, we believe that the EnSCs can be considered as an impactful stem cell for managing effective regenerative angiogenesis in the injured limb tissue microenvironment (**Figure 3**).

Aside from angiogenesis, reconstructing the failed myocytes into the ischemic limb area is introduced as another target along with limb tissue regeneration. In this regard, our team's remarks claimed that the EnSCs seem to be a suitable cellular source for regulating myogenesis in addition to the other well-trialed sources like the MSCs. Various experimental study showed that MSCs could differentiate into skeletal muscle cells and could be a feet source for repair (Gang et al., 2004; Sassoli et al., 2014). Gang et al. (2004) isolated MSCs from umbilical cord blood and induced them to differentiate into skeletal muscle cells. They recommended this stem cell phenotype as a useful tool for muscle-related tissue engineering (Gang et al., 2004). A few years later, Toyoda et al. (2007) described the EnSCs derived from menstrual blood have the remarkable myogenic potential that could allow "rescue" dystrophied myocytes in the MDX model through cell fusion and transdifferentiation (Toyoda et al., 2007). In their work, Cui et al. (2007) showed that endometrial progenitors and menstrual blood-derived mesenchymal stem cells could efficiently transdifferentiate into myoblasts/myocytes, both in *in vitro* and *in vivo* experiments. They expected that the EnSCs could be a major advancement toward cell-based therapies for chronic muscular diseases and muscle injury (Cui et al., 2007). However, based on our investigations, we have a more transcendent view of EnSCs as a powerful tool for myocyte regeneration. In 2009, we carefully hypothesized that EnSCs could be a more suitable option for muscle regeneration cell therapy, based on the properties of EnSCs, including easy accessibility, easy purification, clonogenicity, and maintenance of normal karyotyping in extended passages (Cui et al., 2007). Also in 2014, one of our lab's investigations showed that the EnSCs could serve as a potent myogenic cell source, having a superior differentiation capacity over that of BM-MSCs that was fully supported by Real-Time polymerase chain reaction (PCR) and immunocytochemistry to detect myogenic markers and quantitative-PCR for the upregulation of desmin, myoblast determination protein 1 (MyoD), and troponin T transcripts (Faghihi et al., 2014). All the presented pieces of evidence illustrate that the EnSCs could serve as a proper target for achieving an effective myogenic response in the ischemic limb tissue like the MSCs (**Figure 3**).

The stressful conditions that form early after ischemia has been able to cause cellular death cascades in all limb cell populations as well as neurons. Recreating the degenerated PNS within patients with a large limb injury could be an impactful method for recovering their lost limb functions. This cell therapy research has been focusing on finding a suitable source of stem cells to regenerate the PNS after limb ischemia. Considering the ethical grounds, purity, viability, and tumorigenicity, EnSCs can potentially be a promising source of easily accessible, substantial, and multipotent adult stem cells. In this endeavor, the EnSCs could be used as an alternative source (**Figure 3**). It is well understood that human EnSCs have provided new and effective approaches for neural cell programming. Our experiments reported that neuron-like cells were differentiated from EnSCs by different *in vitro* methods (Navaei-Nigjeh et al., 2014; Mirzaei et al., 2016; Hasanzadeh et al., 2021).

During the course of their research, Meng et al. (2007) have shown that the EnSCs, during their neurogenic differentiation, could express cytoskeletal proteins that include neurofilament-light (NF-L), class III b-tubulin (b3-tub), microtubule associated protein 2 (MAP 2), and oligodendrocyte transcription factor 1 (olig1) as mature markers in the differentiated neuronal-like cell (Meng et al., 2007). Moreover, in 2012, we were able to successfully make neuronal-like cell phenotypes from the EnSCs. Our flow cytometric analysis demonstrated the positive

expression of cell surface markers including CD90, CD105, OCT4, CD44, and negative for CD31, CD34, and CD133. Moreover, our q-PCR and immunocytochemistry evaluation shown expression of the nestin, c-aminobutyric acid (GABA), MAP2, class III β-tubulin (b3-tub), and neurofilament light polypeptide (NF-L) in the EnSCs (Mobarakeh et al., 2012). Following our subsequent projects, Ebrahimi-Barough et al. (2013a) could generate oligodendrocyte progenitor cells (OPCs) from human EnSCs and Asmani et al. (2013) differentiated the OPCs in a 3D culture system (fibrin hydrogel) from EnSCs (Ebrahimi-Barough et al., 2013a; Ebrahimi-Barough et al., 2015). Due to these results, we hypothesized that the activation of the miR-338 cascade plays a central role in the generation of oligodendrocyte cells from the EnSCs (Ebrahimi-Barough et al., 2013b).

To further our studies, we carefully investigated and compared the differentiation potential of the BMMSC and EnSCs to motor neuron-like cells on a nanofibrous scaffold (poly ε-caprolactone) using signaling molecules. Immunostaining and real-time PCR demonstrated expression of beta-tubulin III, islet-1, motor neuron and pancreas homeobox 1 (MNX1), neurofilament-H (NF-H), paired box 6 (Pax6), and choactase-positive motor neurons. We finally concluded that both cells had potential in the differentiation of motor neuron-like cells, but EnSc was superior to BM-MSC (Shirian et al., 2016). During the course of the study, our team was able to differentiate Schwann cells from EnSCs for the first time, using fibrin gel as a 3D culture environment by induction media. Immunocytochemistry confirmed the indicative markers (S100 and P75) for Schwann cells (Bayat et al., 2016). Also, our more recent *in vivo* examination of animal models with PNS disorders (sciatic nerve injury) demonstrated nerve regeneration in rats after the sciatic transaction followed by human EnSCs treatment in a defined nanofiber conduit (Mohamadi et al., 2018). As a result, the motor and sensory integrity were significantly improved in EnSCs-treated animals. All the pieces of evidence given before are able to display the feasibility and efficacy of the EnSC therapy to regenerate the injured limb's PNS networks (**Figure 2**).

## CHALLENGES AND SOLUTIONS

In an effort to develop a more efficient method of stem cell transfusion, scientists have spent more than 13 years doing clinical research on the subject (Lobo et al., 1991). After numerous observations, trial-and-error, and analysis, we have finally reached a breakthrough for stem cell-based therapy approaches, mainly for ischemic disorders. Proper understanding of the most common barriers and challenges is key to achieving an effective stem cell therapy method that could assist in the regeneration of ischemic regions. Based on our experience, these aforementioned barriers can be summarized into three separate steps: 1) stem cell manufacturing, 2) choosing the fittest stem cells based on disease characteristics, and 3) mechanisms of ischemia pathogenesis. In this section, we focus on these main challenges and then explain some practical solutions for overcoming those challenges. **Figure 4** briefly displays the different limitations of ischemic disorder stem cell therapy and presents some efficient solutions for each issue (**Figure 4**).

### Stem Cells Manufacturing

Nowadays, developing technologies for the large-scale production of viable stem cells, and/or cellular products is a determining factor for the expansion of stem cell-based therapy in practice (Ausubel et al., 2011). Utilizing these types of technologies not only has a positive impact on reducing the price of cell therapy for each case, but can also be a solution to overcome ethical and logistical issues found with autologous stem cell therapies (Ausubel et al., 2011). Assuring good quality standards for biopharmaceutical production is done by following the good manufacturing practice (GMP) regulations (Jo et al., 2020). Accordingly, the GMP standards must be considered when manufacturing different cell products, including stem cells and differentiated cells. In this regard, GMP's quality management system is largely used to monitor all the phases of the biopharmaceutical manufacturing process. Based on the GMP instructions, the purpose of the quality control (QC) section of a medical production factory is to guarantee product quality. It is based on a clear association between accurate assessment and critical quality features of the product of interest, including having an identity, being safe, pure, and potential (Viganò et al., 2018). Particular guidelines like the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) identify and explain the as-mentioned matters (Halme and Kessler, 2006; Emea, 2008). Some crucial steps in safety assessments are the evaluation of any microbial contamination (viral, bacterial, and fungal), teratogenic potential, and any biological abnormality in the final cell product (Viganò et al., 2018). These types of accurate quality controls and potentiometry of cell products make GMP guidelines a very powerful tool for the production of safe and efficient stem cells for a cost-effective stem cell therapy approach.

### Selection of an Efficient Stem Cell Type

Identifying and utilizing the most proper types of stem cell production, dosage, and implantation methods is another main issue for managing effective stem cell therapy in ischemic tissues. Achieving this mentioned target is directly related to having a suitable awareness of the ischemia intensity, phase of inflammatory reactions, reperfusion status, and several additional characteristics that appear in ischemic organs (Khodayari et al., 2019). After reviewing the collected information that can be found in **Supplementary Table S1**, it is hypothesized that this recorded controversial outcome, resulting from the stem cell administration, may be linked with a shortage of accurate awareness of the disease status and its possible feedback on the implanted cells. Nowadays, guideline-based medicine has become the mainstay of ischemia treatment (Zabel et al., 2020). Presenting these types of practical, evidence-based guidelines for stem cell therapy of ischemic disorders can be a solution to overcoming this challenge. The availability of clinical data, in particular from randomized controlled clinical trials (RCTs) for a specific query generally leads to the guidelines with overwhelming evidence (de

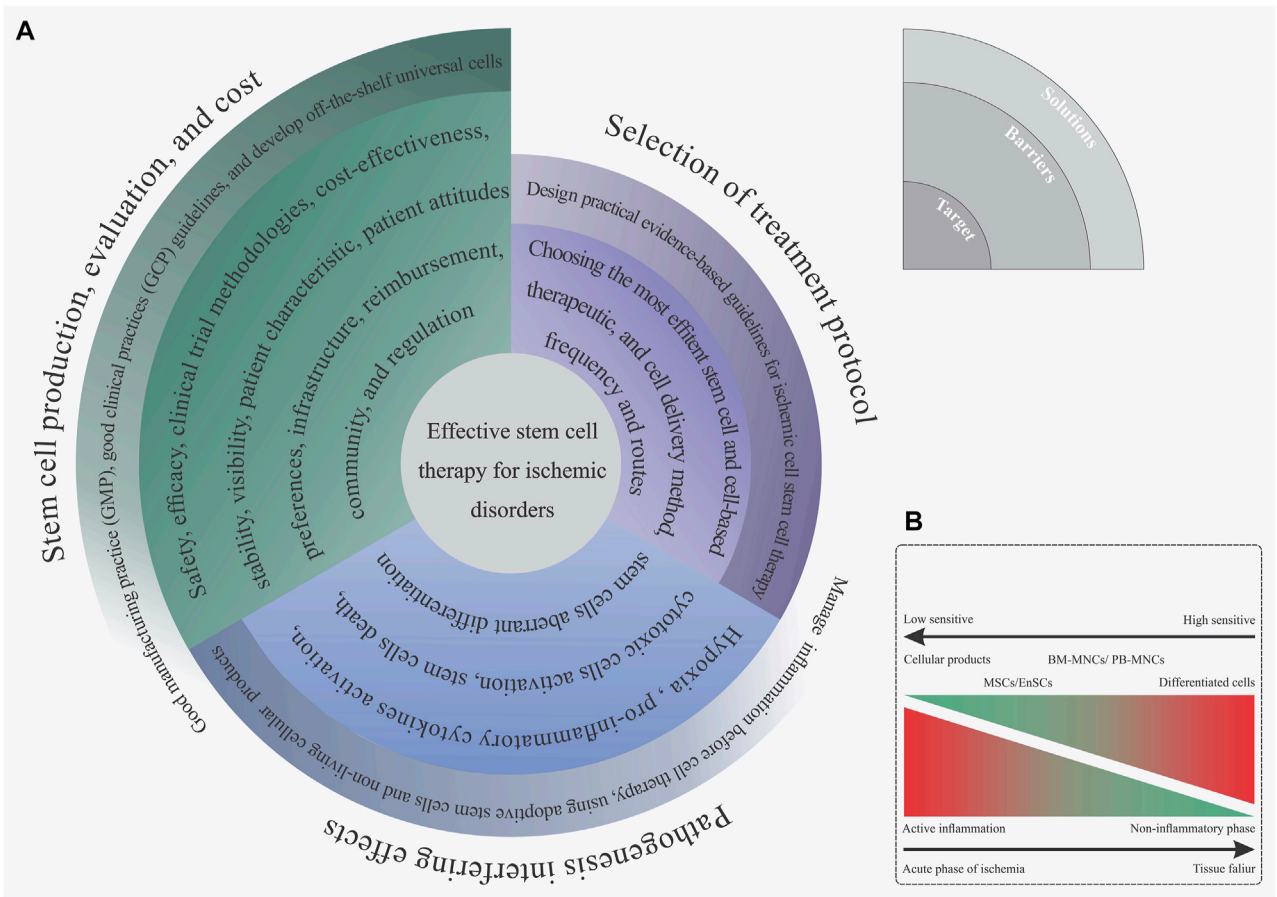


**FIGURE 4 | (A)** Spiral diagram of challenges and solutions toward an effective stem cell therapy for ischemic disorder. **(B)** Complex biological outcome resulting from the ischemia inflammatory microenvironment on the implanted stem cells based on the stem cell type.

Clercq, 2003). Concisely, the RCTs of ischemia stem cell therapy should meet inclusive and exclusive requirements. These criteria provide a wide and enriched study population, leading to expected goals along with different results from statistical and clinical points of view (Burns et al., 2011). Subgroup analyses on the predetermined ones and other post hoc analyses can assist with the identification of the features linked with more advantages, without benefit or damage from stem cell-based therapy, in the study population (Tanniou et al., 2016). So far, the FDA has released some guidelines for cellular and gene therapy approaches (Corsaro et al., 2021); however, none of them refer to ischemia disease treatments. Presenting an evidence-based guideline in order to administer the most efficient stem cell therapy treatment based on the patient's characteristics can be an evolutionary achievement in the field of cell therapy for ischemic diseases.

## Ischemia Pathological Mechanism Adverse Effects

The process of ischemia pathogenesis explains a complex and dynamic biological mechanism that eventually terminates in organ familiarity and dysfunction, as we previously explained in section one, "limb ischemia pathophysiology: pathways and mechanisms." In this regard, the formation of these types of complex biological reactions following ischemia disrupts the implanted stem cells (Khodayari et al., 2019). That means that the development of these mechanisms will not only promote cell death and tissue degeneration in the ischemic organ but can also have a negative biological effect on the implanted cells. In 2019, we hypothesized that the creation of an intense inflammatory response during the acute phase of myocardial ischemia can be a confounding factor in the reduction of efficacy for cell therapy in patients with acute myocardial infarction. Hence, employing types of efficient paraclinical testings in order to obtain an accurate recognition of the disease features and failure characteristics can be an effective approach in future cell therapy measures. In addition to this approach, reducing inflammation before cell therapy as well as stem cell concomitant injection with anti-inflammatory drug therapy or cells with immunoregulatory potential, like MSCs, can be an efficient approach to solving this challenge (Khodayari et al., 2019).

Instead of direct implantation of stem cells, it has been suggested that using extracellular vesicles (exosomes) can be a

new perspective on regenerative medicine and cell-free therapy in ischemia subjects with active inflammation (Anderson et al., 2016; Liang X. et al., 2016; Phinney and Pittenger, 2017). This type of stem cell product is entirely resistant to inflammation pathogenesis (Xia et al., 2019). Direct cell-cell micro-communication is commonly mediated by exosomes through the use of critical functional molecules including nucleotides, proteins, as well as bioactive lipids. Different SCs, for instance, MSCs, secrete the as-mentioned small membrane vesicles (30–100 nm) (Anderson et al., 2016; Liang X. et al., 2016; Phinney and Pittenger, 2017). It is believed that healing after myocardial infarction might be regulated by MSCs-secreted exosomes. A research study was conducted on rat models with acute myocardial infarction (AMI) and proved that MSC-derived exosomes improve the function of the myocardium just after heart injury. This is related to inflammatory microenvironment reprogramming in AMI. Our *ex vivo* investigation in 2019 has suggested that the EnSCs-derived exosome could regulate angiogenesis and vessel formation in the endothelial progenitor cells (Taghdiri Nooshabadi et al., 2019). As a result, we found that this EnSCs-derived exosome angiogenic outcome operates by a dosage-dependent action (Taghdiri Nooshabadi et al., 2019), as evidenced by the dual results observed in drugs and agents as well as atorvastatin (Goodarzi et al., 2020). In summary, once EnSC-derived exosomes hold biological effects similar to their source cells, they could be deemed as new, cell-free, therapeutic candidates. Generally, as angiogenesis increases, the exosomes derived from EnSC indicate the possibility for use in regenerative medicine, particularly for ischemic disorders (Taghdiri Nooshabadi et al., 2019). So far, in addition to targeting treatment of acute ischemic stroke (NCT03384433), different clinical studies have been launched to evaluate the safety and efficacy of stem cell-derived exosomes on different disorders like acute respiratory distress syndrome (NCT04602104, NCT04798716), depression, anxiety, dementias (NCT04202770), periodontitis (NCT04270006), and metastatic pancreas cancer (NCT03608631).

## CONCLUSION AND FUTURE PERSPECTIVE

Destruction of all cellular populations soon after the initiation of ischemia is an inevitable outcome in the ischemic limb regions. Recreation of vascular networks in a stressed tissue or organ is introduced as the first line of stem cell therapy for these patients. However, it appears that through neovascularization, improving blood circulation is not therapeutic enough to recover the ischemic limb function. Theoretically, utilization of a safe stem cell variety with the potential to generate other limb tissue's cellular niches in addition to the endothelial cells, such as the myocytes and PNS cells, dramatically enhances the stem cell therapy effectiveness to promote the limb ischemia patient's function. According to these criteria, although stem cell therapy has offered a closely acceptable output in various experimental and clinical trials, taking advantage of new and more proper stem cell sources will provide us with a suitable treatment for injured limb regeneration. Throughout the last decade, our lab's efforts were to clarify the EnSCs' biology and regeneration potential for improving several degenerative disorders. We remark that although the EnSC niche is derived from an inappropriate tissue, the high potential to generate an array of cellular populations alongside their low immunogenicity will make this lineage a new source of LI regeneration. Using this method, designing translational studies and then measuring the direct regenerative effects of EnSCs on the failed limb are one of our main approaches. However, different limitations and unsolved barriers prevent us from performing an effective regeneration therapy on ischemic diseases using life and autologous stem cells. Nonetheless, to overcome this challenge, researchers should move to develop modern approaches according to prior experiences.It seems to be an adequately safe, efferent, and cost-effective regeneration therapy method for different types of human disorders by: 1) designing a practical evidence-based guideline for stem cell administration, 2) utilizing next-generation regenerative methods, including off-the-shelf universal stem cell and cell-free exosome therapy, and 3) moderating the ischemic limb tissue inflammatory response before cell injection by using common therapeutics seems to be an adequately safe, efferent, and cost-effective regeneration therapy method for different types of human disorders.

## AUTHORS CONTRIBUTIONS

Study design: SK, HK, SE-B, MK, and NA. Conceptualization/original draft preparation: SK, HK, MI. Graphical art works and tables: SK and HK. Review and editing: SE-B, MK, MV, AG, KN, and HM. Review, editing, and supervision: NA and JA.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.834754/full#supplementary-material

## REFERENCES


Abe, Y., Ozaki, Y., Kasuya, J., Yamamoto, K., Ando, J., Sudo, R., et al. (2013). Endothelial Progenitor Cells Promote Directional Three-Dimensional Endothelial Network Formation by Secreting Vascular Endothelial Growth Factor. *PloS One* 8, e82085. doi:10.1371/journal.pone.0082085

Ai, J., Ebrahimi, S., Ai, A., Karimi, R., and Bahrami, N. (2013). Effect of Deforolimus and VEGF on Angiogenesis in Endometrial Stromal Cells Following Three-Dimensional Culture. *Stem Cel. Discov.* 3, 73–75. doi:10.4236/scd.2013.31002

Ai, J., and Ebrahimi, S. (2010). Are Endometrial Stem Cells Novel Tools against Ischemic Heart Failure in Women? a Hypothesis. *Iranian Red Crescent Med. J.* 12. doi:10.4236/scd.2013.31002

Almasri, J., Adusumalli, J., Asi, N., Lakis, S., Alsawas, M., Prokop, L. J., et al. (2019). A Systematic Review and Meta-Analysis of Revascularization Outcomes of Infrainguinal Chronic Limb-Threatening Ischemia. *J. Vasc. Surg.* 69, 126S–136S. doi:10.1016/j.jvs.2018.01.071

Anderson, J. D., Johansson, H. J., Graham, C. S., Vesterlund, M., Pham, M. T., Bramlett, C. S., et al. (2016). Comprehensive Proteomic Analysis of Mesenchymal Stem Cell Exosomes Reveals Modulation of Angiogenesis via Nuclear Factor-KappaB Signaling. *Stem Cells* 34, 601–613. doi:10.1002/stem.2298

Ausubel, L. J., Lopez, P. M., and Couture, L. A. (2011). "GMP Scale-Up and Banking of Pluripotent Stem Cells for Cellular Therapy Applications," in *Human Pluripotent Stem Cells* (Springer), 147–159. doi:10.1007/978-1-61779-201-4_11

Avitabile, D., Crespi, A., Brioschi, C., Parente, V., Toietta, G., Devanna, P., et al. (2011). Human Cord Blood CD34+ Progenitor Cells Acquire Functional Cardiac Properties Through a Cell Fusion Process. *Am. J. Physiology-Heart Circulatory Physiol.* 300, H1875–H1884. doi:10.1152/ajpheart.00523.2010

Bagheri, F., Khori, V., Alizadeh, A. M., Khalighfard, S., Khodayari, S., and Khodayari, H. (2016). Reactive Oxygen Species-Mediated Cardiac-Reperfusion Injury: Mechanisms and Therapies. *Life Sci.* 165, 43–55. doi:10.1016/j.lfs.2016.09.013

Batandier, C., Leverve, X., and Fontaine, E. (2004). Opening of the Mitochondrial Permeability Transition Pore Induces Reactive Oxygen Species Production at the Level of the Respiratory Chain Complex I. *J. Biol. Chem.* 279, 17197–17204. doi:10.1074/jbc.m310329200

Bayat, N., Ebrahimi-Barough, S., Ardakan, M. M. M., Ai, A., Kamyab, A., Babaloo, N., et al. (2016). Differentiation of Human Endometrial Stem Cells into Schwann Cells in Fibrin Hydrogel as 3D Culture. *Mol. Neurobiol.* 53, 7170–7176. doi:10.1007/s12035-015-9574-7

Beeken, L. J., Ting, D. S., and Sidney, L. E. (2021). Potential of Mesenchymal Stem Cells as Topical Immunomodulatory Cell Therapies for Ocular Surface Inflammatory Disorders. *Stem Cell Translational Med.* doi:10.1002/sctm.20-0118

Bender, J. G., Unverzagt, K., Walker, D. E., Lee, W., Smith, S., Williams, S., et al. (1994). Phenotypic Analysis and Characterization of CD34+ Cells from Normal Human Bone Marrow, Cord Blood, Peripheral Blood, and Mobilized Peripheral Blood from Patients Undergoing Autologous Stem Cell Transplantation. *Clin. Immunol. Immunopathology* 70, 10–18. doi:10.1006/clin.1994.1003

Berger, M., Bergers, G., Arnold, B., HäMmerling, G. N. J., and Ganss, R. (2005). Regulator of G-Protein Signaling-5 Induction in Pericytes Coincides with Active Vessel Remodeling During Neovascularization. *Blood* 105, 1094–1101. doi:10.1182/blood-2004-06-2315

Bhansali, S., Dutta, P., Kumar, V., Yadav, M. K., Jain, A., Mudaliar, S., et al. (2017). Efficacy of Autologous Bone Marrow-Derived Mesenchymal Stem Cell and Mononuclear Cell Transplantation in Type 2 Diabetes Mellitus: a Randomized, Placebo-Controlled Comparative Study. *Stem Cell Dev.* 26, 471–481. doi:10.1089/scd.2016.0275

Bockeria, L., Bogin, V., Bockeria, O., Le, T., Alekyan, B., Woods, E. J., et al. (2013). Endometrial Regenerative Cells for Treatment of Heart Failure: A New Stem Cell Enters the Clinic. *J. Translational Med.* 11, 1–8. doi:10.1186/1479-5876-11-56

Bouland, C., Philippart, P., Dequanter, D., Corrillon, F., Loeb, I., Bron, D., et al. (2021). Cross-Talk Between Mesenchymal Stromal Cells (MSCs) and Endothelial Progenitor Cells (EPCs) in Bone Regeneration. *Front. Cel. Developmental Biol.* 9, 1251. doi:10.3389/fcell.2021.674084

Bura, A., Planat-Benard, V., Bourin, P., Silvestre, J.-S., Gross, F., Grolleau, J.-L., et al. (2014). Phase I Trial: The Use of Autologous Cultured Adipose-Derived Stroma/Stem Cells to Treat Patients with Non-Revascularizable Critical Limb Ischemia. *Cytotherapy* 16, 245–257. doi:10.1016/j.jcyt.2013.11.011

Burns, P. B., Rohrich, R. J., and Chung, K. C. (2011). The Levels of Evidence and Their Role in Evidence-Based Medicine. *Plast. Reconstr. Surg.* 128, 305. doi:10.1097/prs.0b013e318219c171

Cabezas, J., Rojas, D., Navarrete, F., Ortiz, R., Rivera, G., Saravia, F., et al. (2018). Equine Mesenchymal Stem Cells Derived from Endometrial or Adipose Tissue Share Significant Biological Properties, but Have Distinctive Pattern of Surface Markers and Migration. *Theriogenology* 106, 93–102. doi:10.1016/j.theriogenology.2017.09.035

Cancelas, J., Querol, S., Canals, C., Picon, M., Azqueta, C., Sola, C., et al. (1998). Peripheral Blood CD34+ Cell Immunomagnetic Selection in Breast Cancer Patients: Effect on Hematopoietic Progenitor Content and Hematologic Recovery after High-Dose Chemotherapy and Autotransplantation. *Transfusion* 38, 1063–1070. doi:10.1046/j.1537-2995.1998.38111299056318.x

Canosa, S., Moggio, A., Brossa, A., Pittatore, G., Marchino, G., Leoncini, S., et al. (2017). Angiogenic Properties of Endometrial Mesenchymal Stromal Cells in Endothelial Co-Culture: an *In Vitro* Model of Endometriosis. *MHR: Basic Sci. Reprod. Med.* 23, 187–198. doi:10.1093/molehr/gax006

Chan, R. W., Schwab, K. E., and Gargett, C. E. (2004). Clonogenicity of Human Endometrial Epithelial and Stromal Cells. *Biol. Reprod.* 70, 1738–1750. doi:10.1095/biolreprod.103.024109

Chegini, N., Rossi, M. J., and Masterson, B. J. (1992). Platelet-Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), and EGF and PDGF Beta-Receptors in Human Endometrial Tissue: Localization and *In Vitro* Action. *Endocrinology* 130, 2373–2385. doi:10.1210/en.130.4.2373

Cheng, A. J., Yamada, T., Rassier, D. E., Andersson, D. C., Westerblad, H., and Lanner, J. T. (2016). Reactive Oxygen/Nitrogen Species and Contractile Function in Skeletal Muscle during Fatigue and Recovery. *J. Physiol.* 594, 5149–5160. doi:10.1113/jp270650

Cheng, Y., Li, L., Wang, D., Guo, Q., He, Y., Liang, T., et al. (2017). Characteristics of Human Endometrium-Derived Mesenchymal Stem Cells and Their Tropism to Endometriosis. *Stem Cell Int.* doi:10.1155/2017/4794827

Cnop, M., Welsh, N., Jonas, J.-C., Jörns, A., Lenzen, S., and Eizirik, D. L. (2005). Mechanisms of Pancreatic β-Cell Death in Type 1 and Type 2 Diabetes: Many Differences, Few Similarities. *Diabetes* 54, S97–S107. doi:10.2337/diabetes.54.suppl_2.s97

Corsaro, B., Yang, T.-Y., Murphy, R., Sonderegger, I., Exley, A., Bertholet, S., et al. (2021). 2020 White Paper on Recent Issues in Bioanalysis: Vaccine Assay Validation, qPCR Assay Validation, QC for CAR-T Flow Cytometry, NAb Assay Harmonization and ELISpot Validation (Part 3–Recommendations on Immunogenicity Assay Strategies, NAb Assays, Biosimilars and FDA/EMA Immunogenicity Guidance/Guideline, Gene & Cell Therapy and Vaccine Assays). *Bioanalysis* 13, 415–463. doi:10.4155/bio-2021-0007

Cortés-Araya, Y., Amilon, K., Rink, B. E., Black, G., Lisowski, Z., Donadeu, F. X., et al. (2018). Comparison of Antibacterial and Immunological Properties of Mesenchymal Stem/Stromal Cells from Equine Bone Marrow, Endometrium, and Adipose Tissue. *Stem Cell Dev.* 27, 1518–1525. doi:10.1089/scd.2017.0241

Cosentino-Gomes, D., Rocco-Machado, N., and Meyer-Fernandes, J. R. (2012). Cell Signaling through Protein Kinase C Oxidation and Activation. *Int. J. Mol. Sci.* 13, 10697–10721. doi:10.3390/ijms130910697

Cui, C.-H., Uyama, T., Miyado, K., Terai, M., Kyo, S., Kiyono, T., et al. (2007). Menstrual Blood-Derived Cells Confer Human Dystrophin Expression in the Murine Model of Duchenne Muscular Dystrophy via Cell Fusion and Myogenic Transdifferentiation. *Mol. Biol. Cel.* 18, 1586–1594. doi:10.1091/mbc.e06-09-0872

Curtis, E., Martin, J. R., Gabel, B., Sidhu, N., Rzesiewicz, T. K., Mandeville, R., et al. (2018). A First-In-Human, Phase I Study of Neural Stem Cell Transplantation for Chronic Spinal Cord Injury. *Cell Stem Cell* 22, 941–950. e946. doi:10.1016/j.stem.2018.05.014

Daltro, G. C., Fortuna, V., De Souza, E. S., Salles, M. M., Carreira, A. C., Meyer, R., et al. (2015). Efficacy of Autologous Stem Cell-Based Therapy for Osteonecrosis of the Femoral Head in Sickle Cell Disease: A Five-Year Follow-Up Study. *Stem Cel. Res. Ther.* 6, 1–18. doi:10.1186/s13287-015-0105-2

Das, A. K., Abdullah, B. J. J. B., Dhillon, S., Vijanari, A., Anoop, C., and Gupta, P. K. (2013). Intra-Arterial Allogeneic Mesenchymal Stem Cells for Critical Limb

Ischemia Are Safe and Efficacious: Report of a Phase I Study. *World J. Surg.* 37, 915–922. doi:10.1007/s00268-012-1892-6

De Clercq, P. (2003). *Guideline-Based Decision Support in Medicine*. Citeseer.

Dubsky, M., Jirkovska, A., Bem, R., Fejfarova, V., Pagacova, L., Sixta, B., et al. (2013). Both Autologous Bone Marrow Mononuclear Cell and Peripheral Blood Progenitor Cell Therapies Similarly Improve Ischaemia in Patients with Diabetic Foot in Comparison with Control Treatment. *Diabetes/Metabolism Res. Rev.* 29, 369–376. doi:10.1002/dmrr.2399

Ebrahimi-Barough, S., Hoveizi, E., Norouzi Javidan, A., and Ai, J. (2015). Investigating the Neuroglial Differentiation Effect of Neuroblastoma Conditioned Medium in Human Endometrial Stem Cells Cultured on 3D Nanofibrous Scaffold. *J. Biomed. Mater. Res. A* 103, 2621–2627. doi:10.1002/jbm.a.35397

Ebrahimi-Barough, S., Kouchesfahani, H. M., Ai, J., and Massumi, M. (2013a). Differentiation of Human Endometrial Stromal Cells into Oligodendrocyte Progenitor Cells (OPCs). *J. Mol. Neurosci.* 51, 265–273. doi:10.1007/s12031-013-9957-z

Ebrahimi-Barough, S., Massumi, M., Kouchesfahani, H. M., and Ai, J. (2013b). Derivation of Pre-Oligodendrocytes from Human Endometrial Stromal Cells by Using Overexpression of microRNA 338. *J. Mol. Neurosci.* 51, 337–343. doi:10.1007/s12031-013-0101-x

Emadedin, M., Aghdami, N., Taghiyar, L., Fazeli, R., Moghadasali, R., Jahangir, S., et al. (2012). Intra-Articular Injection of Autologous Mesenchymal Stem Cells in Six Patients with Knee Osteoarthritis. *Arch. Iranian Med.* 15, 422.

Emadedin, M., Labibzadeh, N., Liastani, M. G., Karimi, A., Jaroughi, N., Bolurieh, T., et al. (2018). Intra-Articular Implantation of Autologous Bone Marrow–Derived Mesenchymal Stromal Cells to Treat Knee Osteoarthritis: a Randomized, Triple-Blind, Placebo-Controlled Phase 1/2 Clinical Trial. *Cytotherapy* 20, 1238–1246. doi:10.1016/j.jcyt.2018.08.005

Emea, E. (2008). *Guideline on Human Cell-Based Medicinal Products*. Great Britain): Official Guideline of the European Medicines Agency: EMA London.

Esfandiari, N., Ai, J., Nazemian, Z., Javed, M. H., Gotlieb, L., and Casper, R. F. (2007a). Expression of Glycodelin and Cyclooxygenase-2 in Human Endometrial Tissue Following Three-Dimensional Culture. *Am. J. Reprod. Immunol.* 57, 49–54. doi:10.1111/j.1600-0897.2006.00445.x

Esfandiari, N., Khazaei, M., Ai, J., Bielecki, R., Gotlieb, L., Ryan, E., et al. (2007b). Effect of a Statin on an *In Vitro* Model of Endometriosis. *Fertil. Sterility* 87, 257–262. doi:10.1016/j.fertnstert.2006.06.040

Esfandiari, N., Khazaei, M., Ai, J., Nazemian, Z., Jolly, A., Casper, R. F., et al. (2008). Angiogenesis Following Three-Dimensional Culture of Isolated Human Endometrial Stromal Cells Revascularization and Endothelial Progenitor Cells in Stroke. *Am. J. Physiology-Cell Physiol.* 315, C664–C674.

Esquiva, G., Grayston, A., and Rosell, A. (2018). Revascularization and Endothelial Progenitor Cells in Stroke. *Am. J. Physiology-Cell Physiol.* 315, C664–C674. doi:10.1152/ajpcell.00200.2018

Evans, J., Salamonsen, L. A., Winship, A., and Menkhorst, E. (2016). PFertile Ground: Human Endometrial Programming and Lessons in Health and Disease. *Nat. Rev. Endocrinol.* 12, 654–667. doi:10.1038/nrendo.2016.116

Faghihi, F., Jalali, H., Parivar, K., Kajbafzadeh, A., Roozafzoon, R., and Somayeh, E.-B. (2014). Evaluation of Differentiation Potential of Endometrial-Versus Bone Marrow Drived Mesenchymal Stem Cells into Myoblast-Like Cells. *IJCRLS* 4, 3992–3997.

Fowkes, F., Aboyans, V., Fowkes, F., Mcdermott, M., and Sampson, U. (2017). Peripheral Artery Disease: Epidemiology and Global Perspectives. *Nat. Rev. Cardiol.* 14, 156–170. doi:10.1038/nrcardio.2016.179

Friedrich, E. B., Walenta, K., Scharlau, J., Nickenig, G., and Werner, N. (2006). CD34−/CD133+/VEGFR-2+ Endothelial Progenitor Cell Subpopulation with Potent Vasoregenerative Capacities. *Circ. Res.* 98, e20–e25. doi:10.1161/01.RES.0000205765.28940.93

Fuentealba, L. C., Obernier, K., and Alvarez-Buylla, A. (2012). Adult Neural Stem Cells Bridge Their Niche. *Cell Stem Cell* 10, 698–708. doi:10.1016/j.stem.2012.05.012

Gang, E. J., Jeong, J. A., Hong, S. H., Hwang, S. H., Kim, S. W., Yang, I. H., et al. (2004). Skeletal Myogenic Differentiation of Mesenchymal Stem Cells Isolated from Human Umbilical Cord Blood. *Stem Cells* 22, 617–624. doi:10.1634/stemcells.22-4-617

Gao, W., Chen, D., Liu, G., and Ran, X. (2019). Autologous Stem Cell Therapy for Peripheral Arterial Disease: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Stem Cel. Res. Ther.* 10, 1–14. doi:10.1186/s13287-019-1254-5

Gargett, C. E., Schwab, K. E., Zillwood, R. M., Nguyen, H. P., and Wu, D. (2009). Isolation and Culture of Epithelial Progenitors and Mesenchymal Stem Cells from Human Endometrium. *Biol. Reprod.* 80, 1136–1145. doi:10.1095/biolreprod.108.075226

Ghazizadeh, Z., Fattahi, F., Mirzaei, M., Bayersaikhan, D., Lee, J., Chae, S., et al. (2018). Prospective Isolation of ISL1+ Cardiac Progenitors from Human ESCs for Myocardial Infarction Therapy. *Stem Cel. Rep.* 10, 848–859. doi:10.1016/j.stemcr.2018.01.037

Giudice, L. C. (1994). Growth Factors and Growth Modulators in Human Uterine Endometrium: Their Potential Relevance to Reproductive Medicine. *Fertil. Sterility* 61, 1–17. doi:10.1016/s0015-0282(16)56447-4

Gong, M., Yu, B., Wang, J., Wang, Y., Liu, M., Paul, C., et al. (2017). Mesenchymal Stem Cells Release Exosomes that Transfer miRNAs to Endothelial Cells and Promote Angiogenesis. *Oncotarget* 8, 45200. doi:10.18632/oncotarget.16778

Goodarzi, A., Khanmohammadi, M., Ai, A., Khodayari, H., Ai, A., Farahani, M. S., et al. (2020). Simultaneous Impact of Atorvastatin and Mesenchymal Stem Cells for Glioblastoma Multiform Suppression in Rat Glioblastoma Multiform Model. *Mol. Biol. Rep.* 47, 7783–7795. doi:10.1007/s11033-020-05855-z

Gupta, P. K., Chullikana, A., Parakh, R., Desai, S., Das, A., Gottipamula, S., et al. (2013). A Double Blind Randomized Placebo Controlled Phase I/II Study Assessing the Safety and Efficacy of Allogeneic Bone Marrow Derived Mesenchymal Stem Cell in Critical Limb Ischemia. *J. Translational Med.* 11, 1–11. doi:10.1186/1479-5876-11-143

Gupta, P. K., Krishna, M., Chullikana, A., Desai, S., Murugesan, R., Dutta, S., et al. (2017). Administration of Adult Human Bone Marrow-Derived, Cultured, Pooled, Allogeneic Mesenchymal Stromal Cells in Critical Limb Ischemia Due to Buerger's Disease: Phase II Study Report Suggests Clinical Efficacy. *Stem Cell Translational Med.* 6, 689–699. doi:10.5966/sctm.2016-0237

Halme, D. G., and Kessler, D. A. (2006). FDA Regulation of Stem-Cell-Based Therapies. *New Engl. J. Med.* 355, 1730. doi:10.1056/nejmhpr063086

Harbom, L. J., Michel, N., and Mcconnell, M. J. (2018). Single-Cell Analysis of Diversity in Human Stem Cell-Derived Neurons. *Cel. Tissue Res.* 371, 171–179. doi:10.1007/s00441-017-2728-3

Hasanzadeh, E., Ebrahimi-Barough, S., Mahmoodi, N., Mellati, A., Nekounam, H., Basiri, A., et al. (2021). Defining the Role of 17β-Estradiol in Human Endometrial Stem Cells Differentiation into Neuron-Like Cells. *Cel. Biol. Int.* 45, 140–153. doi:10.1002/cbin.11478

Hashemian, S.-M. R., Aliannejad, R., Zarrabi, M., Soleimani, M., Vosough, M., Hosseini, S.-E., et al. (2021). Mesenchymal Stem Cells Derived from Perinatal Tissues for Treatment of Critically Ill COVID-19-Induced ARDS Patients: A Case Series. *Stem Cel. Res. Ther.* 12, 1–12. doi:10.1186/s13287-021-02165-4

Heath, H., Kudo, H., Renney, G., Ward, M., Cutillas, P., Goldin, R., et al. (2018). *The Pathogenic Role of Stat2 in Inflammation Is Independent of Canonical Interferon Signaling and Is Mediated by TNFa*.

Heldman, A. W., Difede, D. L., Fishman, J. E., Zambrano, J. P., Trachtenberg, B. H., Karantalis, V., et al. (2014). Transendocardial Mesenchymal Stem Cells and Mononuclear Bone Marrow Cells for Ischemic Cardiomyopathy: The TAC-HFT Randomized Trial. *Jama* 311, 62–73. doi:10.1001/jama.2013.282909

Heo, W., Jun, H. J., Do Kyun Kang, H.-K. M., Hwang, Y.-H., Kim, J. Y., and Nam, K. H. (2017). Acute Limb Ischemia and Coronary Artery Disease in a Case of Kimura's Disease. *Korean J. Thorac. Cardiovasc. Surg.* 50, 114. doi:10.5090/kjtcs.2017.50.2.114

Hicks, C. W., and Selvin, E. (2019). Epidemiology of Peripheral Neuropathy and Lower Extremity Disease in Diabetes. *Curr. Diabetes Rep.* 19, 1–8. doi:10.1007/s11892-019-1212-8

Hong, S. H., Gang, E. J., Jeong, J. A., Ahn, C., Hwang, S. H., Yang, I. H., et al. (2005). *In Vitro* Differentiation of Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells into Hepatocyte-Like Cells. *Biochem. Biophysical Res. Commun.* 330, 1153–1161. doi:10.1016/j.bbrc.2005.03.086

Howangyin, K.-Y., Loinard, C., Bakker, W., Guérin, C. L., Vilar, J., D'audigier, C., et al. (2014). HIF-Prolyl Hydroxylase 2 Inhibition Enhances the Efficiency of Mesenchymal Stem Cell-Based Therapies for the Treatment of Critical Limb Ischemia. *Stem Cells* 32, 231–243. doi:10.1002/stem.1540

Huang, W. J., Chen, W. W., and Zhang, X. (2016). Glioblastoma Multiforme: Effect of Hypoxia and Hypoxia Inducible Factors on Therapeutic Approaches. *Oncol. Lett.* 12, 2283–2288. doi:10.3892/ol.2016.4952

Indo, H. P., Davidson, M., Yen, H.-C., Suenaga, S., Tomita, K., Nishii, T., et al. (2007). Evidence of ROS Generation by Mitochondria in Cells with Impaired Electron Transport Chain and Mitochondrial DNA Damage. *Mitochondrion* 7, 106–118. doi:10.1016/j.mito.2006.11.026

Jaluvka, F., Ihnat, P., Madaric, J., Vrtkova, A., Janosek, J., and Prochazka, V. (2020). Current Status of Cell-Based Therapy in Patients with Critical Limb Ischemia. *Int. J. Mol. Sci.* 21, 8999. doi:10.3390/ijms21238999

Jo, H.-Y., Han, H.-W., Jung, I., Ju, J. H., Park, S.-J., Moon, S., et al. (2020). Development of Genetic Quality Tests for Good Manufacturing Practice-Compliant Induced Pluripotent Stem Cells and Their Derivatives. *Scientific Rep.* 10, 1–15. doi:10.1038/s41598-020-60466-9

Johnson, G. L., Henry, T. D., Povsic, T. J., Losordo, D. W., Garberich, R. F., Stanberry, L. I., et al. (2020). CD34+ Cell Therapy Significantly Reduces Adverse Cardiac Events, Health Care Expenditures, and Mortality in Patients with Refractory Angina. *Stem Cell Translational Med.* 9, 1147–1152. doi:10.1002/sctm.20-0046

Johnson, T., Zhao, L., Manuel, G., Taylor, H., and Liu, D. (2019). Approaches to Therapeutic Angiogenesis for Ischemic Heart Disease. *J. Mol. Med.* 97, 141–151. doi:10.1007/s00109-018-1729-3

Kalladka, D., Sinden, J., Pollock, K., Haig, C., Mclean, J., Smith, W., et al. (2016). Human Neural Stem Cells in Patients with Chronic Ischaemic Stroke (PISCES): a Phase 1, First-In-Man Study. *The Lancet* 388, 787–796. doi:10.1016/s0140-6736(16)30513-x

Kamezaki, K., Shimoda, K., Numata, A., Haro, T., Kakumitsu, H., Yoshie, M., et al. (2005). Roles of Stat3 and ERK in G-CSF Signaling. *Stem Cells* 23, 252–263. doi:10.1634/stemcells.2004-0173a

Kaneko, N., Kako, E., and Sawamoto, K. (2011). Prospects and Limitations of Using Endogenous Neural Stem Cells for Brain Regeneration. *Genes* 2, 107–130. doi:10.3390/genes2010107

Kawamoto, A., Katayama, M., Handa, N., Kinoshita, M., Takano, H., Horii, M., et al. (2009). Intramuscular Transplantation of G-CSF-Mobilized CD34+ Cells in Patients with Critical Limb Ischemia: A Phase I/IIa, Multicenter, Single-Blinded, Dose-Escalation Clinical Trial. *Stem Cells* 27, 2857–2864. doi:10.1002/stem.207

Keating, A. (2012). Mesenchymal Stromal Cells: New Directions. *Cell Stem Cell* 10, 709–716. doi:10.1016/j.stem.2012.05.015

Khademi, F., Verdi, J., Soleimani, M., Roozafzoon, R., Keshel, S. H., Raeisossadati, R., et al. (2014). Human Endometrial Adult Stem Cells Can Be Differentiated into Hepatocyte Cells. *J. Med. Hypotheses Ideas* 8, 30–33. doi:10.1016/j.jmhi.2013.07.004

Khalighfard, S., Khori, V., Alizadeh, A. M., Vahabzadeh, G., Tajaldini, M., Sedighi, S., et al. (2021). Dual Effects of Atorvastatin on Angiogenesis Pathways in the Differentiation of Mesenchymal Stem Cells. *Eur. J. Pharmacol.*, 174281. doi:10.1016/j.ejphar.2021.174281

Khodayari, H., Khodayari, S., Ebrahimi, E., Hadjilooei, F., Vesovic, M., Mahmoodzadeh, H., et al. (2021a). Stem Cells-Derived Natural Killer Cells for Cancer Immunotherapy: Current Protocols, Feasibility, and Benefits of *Ex Vivo* Generated Natural Killer Cells in Treatment of Advanced Solid Tumors. *Cancer Immunol. Immunother.* 70, 3369–3395. doi:10.1007/s00262-021-02975-8

Khodayari, H., Khodayari, S., Khalighfard, S., Tahmasebifar, A., Tajaldini, M., Poorkhani, A., et al. (2021b). Gamma-Radiated Immunosuppressed Tumor Xenograft Mice Can Be a New Ideal Model in Cancer Research. *Scientific Rep.* 11, 1–13. doi:10.1038/s41598-020-80428-5

Khodayari, S., Khodayari, H., Amiri, A. Z., Eslami, M., Farhud, D., Hescheler, J., et al. (2019). Inflammatory Microenvironment of Acute Myocardial Infarction Prevents Regeneration of Heart with Stem Cells Therapy. *Cell Physiol. Biochem.* 53, 887–909. doi:10.33594/000000180

Kim, J.-Y., Morgan, M., Kim, D.-G., Lee, J.-Y., Bai, L., Lin, Y., et al. (2011). Tnfα-Induced Noncanonical NF-Kb Activation Is Attenuated by RIP1 Through Stabilization of TRAF2. *J. Cel. Sci.* 124, 647–656. doi:10.1242/jcs.075770

Kim, J., Kim, B., Kim, S., Lee, Y. I., Kim, J., and Lee, J. H. (2020). The Effect of Human Umbilical Cord Blood–Derived Mesenchymal Stem Cell Media Containing Serum on Recovery After Laser Treatment: A Double-Blinded, Randomized, Split-Face Controlled Study. *J. Cosmet. Dermatol.* 19, 651–656. doi:10.1111/jocd.13063

Kim, S. H., Cho, J. H., Lee, Y. H., Lee, J. H., Kim, S. S., Kim, M. Y., et al. (2018). Improvement in Left Ventricular Function with Intracoronary Mesenchymal Stem Cell Therapy in a Patient with Anterior Wall ST-Segment Elevation Myocardial Infarction. *Cardiovasc. Drugs Ther.* 32, 329–338. doi:10.1007/s10557-018-6804-z

Kinnaird, T., Stabile, E., Burnett, M., Shou, M., Lee, C., Barr, S., et al. (2004). Local Delivery of Marrow-Derived Stromal Cells Augments Collateral Perfusion Through Paracrine Mechanisms. *Circulation* 109, 1543–1549. doi:10.1161/01.cir.0000124062.31102.57

Kinoshita, M., Fujita, Y., Katayama, M., Baba, R., Shibakawa, M., Yoshikawa, K., et al. (2012). Long-Term Clinical Outcome after Intramuscular Transplantation of Granulocyte colony Stimulating Factor-Mobilized CD34 Positive Cells in Patients with Critical Limb Ischemia. *Atherosclerosis* 224, 440–445. doi:10.1016/j.atherosclerosis.2012.07.031

Krenning, G., Strate, B. V. D., Schipper, M., Van Seijen, X. G. Y., Fernandes, B. C., Van Luyn, M. J., et al. (2009). CD34+ Cells Augment Endothelial Cell Differentiation of CD14+ Endothelial Progenitor Cells *In Vitro*. *J. Cell. Mol. Med.* 13, 2521–2533. doi:10.1111/j.1582-4934.2008.00479.x

Kudo, F., Nishibe, T., Nishibe, M., and Yasuda, K. (2003). Autologous Transplantation of Peripheral Blood Endothelial Progenitor Cells (CD34$^{-}$ Sup+) for Therapeutic Angiogenesis in Patients with Critical Limb Ischemia. *Int. angiology* 22, 344.

Lambert, M., and Belch, J. (2013). Medical Management of Critical Limb Ischaemia: Where Do We Stand Today? *J. Intern. Med.* 274, 295–307. doi:10.1111/joim.12102

Laurence, J., Baptista, P., and Atala, A. (2015). *Translating Regenerative Medicine to the Clinic*. Academic Press.

Lee, F.-Y., Chen, Y.-L., Sung, P.-H., Ma, M.-C., Pei, S.-N., Wu, C.-J., et al. (2015). Intracoronary Transfusion of Circulation-Derived CD34+ Cells Improves Left Ventricular Function in Patients with End-Stage Diffuse Coronary Artery Disease Unsuitable for Coronary Intervention. *Read. Online Crit. Care Med. So.y Cri.l Care Me.e* 43, 2117–2132. doi:10.1097/ccm.0000000000001138

Lee, H. B., Yu, M. R., Song, J. S., and Ha, H. (2004). Reactive Oxygen Species Amplify Protein Kinase C Signaling in High Glucose-Induced Fibronectin Expression by Human Peritoneal Mesothelial Cells. *Kidney Int.* 65, 1170–1179. doi:10.1111/j.1523-1755.2004.00491.x

Leslie, N. R., Lindsay, Y., Ross, S. H., and Downes, C. P. (2004). Redox Regulation of Phosphatase Function. *Biochem. Soc. Trans.* 32, 1018–1020. doi:10.1042/BST0321018

Levy, M. L., Crawford, J. R., Dib, N., Verkh, L., Tankovich, N., and Cramer, S. C. (2019). Phase I/II Study of Safety and Preliminary Efficacy of Intravenous Allogeneic Mesenchymal Stem Cells in Chronic Stroke. *Stroke* 50, 2835–2841. doi:10.1161/strokeaha.119.026318

Li, S., Zhou, B., and Han, Z. C. (2006). Therapeutic Neovascularization by Transplantation of Mobilized Peripheral Blood Mononuclear Cells for Limb Ischemia. *Thromb. Haemost.* 95, 301–311. doi:10.1160/th05-06-0442

Liang, J., Zhang, H., Kong, W., Deng, W., Wang, D., Feng, X., et al. (2018). Safety Analysis in Patients with Autoimmune Disease Receiving Allogeneic Mesenchymal Stem Cells Infusion: A Long-Term Retrospective Study. *Stem Cel. Res. Ther.* 9, 1–10. doi:10.1186/s13287-018-1053-4

Liang, T. W., Jester, A., Motaganahalli, R. L., Wilson, M. G., G'sell, P., Akingba, G. A., et al. (2016a). Autologous Bone Marrow Mononuclear Cell Therapy for Critical Limb Ischemia Is Effective and Durable. *J. Vasc. Surg.* 63, 1541–1545. doi:10.1016/j.jvs.2016.01.022

Liang, X., Zhang, L., Wang, S., Han, Q., and Zhao, R. C. (2016b). Exosomes Secreted by Mesenchymal Stem Cells Promote Endothelial Cell Angiogenesis by Transferring miR-125a. *J. Cel. Sci.* 129, 2182–2189. doi:10.1242/jcs.170373

Liotta, F., Annunziato, F., Castellani, S., Boddi, M., Alterini, B., Castellini, G., et al. (2018). Therapeutic Efficacy of Autologous Non-Mobilized Enriched Circulating Endothelial Progenitors in Patients with Critical Limb Ischemia—the SCELTA Trial. *Circ. J.* 82, 1688–1698. doi:10.1253/circj.cj-17-0720

Liu, X., Fu, X., Dai, G., Wang, X., Zhang, Z., Cheng, H., et al. (2017). Comparative Analysis of Curative Effect of Bone Marrow Mesenchymal Stem Cell and Bone Marrow Mononuclear Cell Transplantation for Spastic Cerebral Palsy. *J. Translational Med.* 15, 1–9. doi:10.1186/s12967-017-1149-0

Lobo, F., Kessinger, A., Landmark, J., Smith, D., Weisenburger, D., Wigton, R., et al. (1991). Addition of Peripheral Blood Stem Cells Collected without Mobilization Techniques to Transplanted Autologous Bone Marrow Did Not Hasten

Marrow Recovery Following Myeloablative Therapy. *Bone Marrow Transplant.* 8, 389–392.

Lu, D., Jiang, Y., Deng, W., Zhang, Y., Liang, Z., Wu, Q., et al. (2019). Long-Term Outcomes of BMMSC Compared with BMMNC for Treatment of Critical Limb Ischemia and Foot Ulcer in Patients with Diabetes. *Cel. Transplant.* 28, 645–652. doi:10.1177/0963689719835177

Mafi, P., Hindocha, S., Mafi, R., Griffin, M., and Khan, W. (2011). Suppl 2: Adult Mesenchymal Stem Cells and Cell Surface Characterization-A Systematic Review of the Literature. *Open Orthopaedics J.* 5, 253. doi:10.2174/1874325001105010253

Makhlough, A., Shekarchian, S., Moghadasali, R., Einollahi, B., Dastgheib, M., Janbabaee, G., et al. (2018). Bone Marrow–Mesenchymal Stromal Cell Infusion in Patients with Chronic Kidney Disease: A Safety Study with 18 Months of Follow-Up. *Cytotherapy* 20, 660–669. doi:10.1016/j.jcyt.2018.02.368

Makhlough, A., Shekarchian, S., Moghadasali, R., Einollahi, B., Hosseini, S. E., Jaroughi, N., et al. (2017). Safety and Tolerability of Autologous Bone Marrow Mesenchymal Stromal Cells in ADPKD Patients. *Stem Cel. Res. Ther.* 8, 1–11. doi:10.1186/s13287-017-0557-7

Mardanpour, P., Nayernia, K., Khodayari, S., Khodayari, H., Molcanyi, M., and Hescheler, J. (2019). Application of Stem Cell Technologies to Regenerate Injured Myocardium and Improve Cardiac Function. *Cel. Physiol. Biochem.* 53, 101–120. doi:10.33594/000000124

Matsiko, A., Levingstone, T. J., and O'brien, F. J. (2013). Advanced Strategies for Articular Cartilage Defect Repair. *Materials* 6, 637–668. doi:10.3390/ma6020637

Meng, X., Ichim, T. E., Zhong, J., Rogers, A., Yin, Z., Jackson, J., et al. (2007). Endometrial Regenerative Cells: A Novel Stem Cell Population. *J. Translational Med.* 5, 1–10. doi:10.1186/1479-5876-5-57

Mirzaei, E., Ai, J., Ebrahimi-Barough, S., Verdi, J., Ghanbari, H., and Faridi-Majidi, R. (2016). The Differentiation of Human Endometrial Stem Cells into Neuron-Like Cells on Electrospun PAN-Derived Carbon Nanofibers with Random and Aligned Topographies. *Mol. Neurobiol.* 53, 4798–4808. doi:10.1007/s12035-015-9410-0

Mobarakeh, Z. T., Ai, J., Yazdani, F., Sorkhabadi, S. M. R., Ghanbari, Z., Javidan, A. N., et al. (2012). Human Endometrial Stem Cells as a New Source for Programming to Neural Cells. *Cel. Biol. Int. Rep.* 19, 7–14. doi:10.1042/cbr20110009

Mohamadi, F., Ebrahimi-Barough, S., Nourani, M. R., Mansoori, K., Salehi, M., Alizadeh, A. A., et al. (2018). Enhanced Sciatic Nerve Regeneration by Human Endometrial Stem Cells in an Electrospun Poly (ε-Caprolactone)/Collagen/NBG Nerve Conduit in Rat. *Artif. Cell Nanomedicine, Biotechnol.* 46, 1731–1743. doi:10.1080/21691401.2017.1391823

Mohamadnejad, M., Vosough, M., Moossavi, S., Nikfam, S., Mardpour, S., Akhlaghpoor, S., et al. (2016). Intraportal Infusion of Bone Marrow Mononuclear or CD133+ Cells in Patients with Decompensated Cirrhosis: a Double-blind Randomized Controlled Trial. *Stem Cell translational Med.* 5, 87–94. doi:10.5966/sctm.2015-0004

Mohamed, S. A., Howard, L., Mcinerney, V., Hayat, A., Krawczyk, J., Naughton, S., et al. (2020). Autologous Bone Marrow Mesenchymal Stromal Cell Therapy for "No-option" Critical Limb Ischemia Is Limited by Karyotype Abnormalities. *Cytotherapy* 22, 313–321. doi:10.1016/j.jcyt.2020.02.007

Molavi, B., Zafarghandi, M., Aminizadeh, E., Hosseini, S. E., Mirzayi, H., Arab, L., et al. (2016). Safety and Efficacy of Repeated Bone Marrow Mononuclear Cell Therapy in Patients with Critical Limb Ischemia in a Pilot Randomized Controlled Trial. *Arch. Iranian Med.* 19, 388–396.

Moniche, F., Escudero, I., Zapata-Arriaza, E., Usero-Ruiz, M., Prieto-León, M., De La Torre, J., et al. (2015). Intra-arterial Bone Marrow Mononuclear Cells (BM-MNCs) Transplantation in Acute Ischemic Stroke (IBIS Trial): Protocol of a Phase II, Randomized, Dose-Finding, Controlled Multicenter Trial. *Int. J. Stroke* 10, 1149–1152. doi:10.1111/ijs.12520

Muir, K. W., Bulters, D., Willmot, M., Sprigg, N., Dixit, A., Ward, N., et al. (2020). Intracerebral Implantation of Human Neural Stem Cells and Motor Recovery after Stroke: Multicentre Prospective Single-Arm Study (PISCES-2). *J. Neurol. Neurosurg. Psychiatry* 91, 396–401. doi:10.1136/jnnp-2019-322515

Murphy, M. P., Wang, H., Patel, A. N., Kambhampati, S., Angle, N., Chan, K., et al. (2008). Allogeneic Endometrial Regenerative Cells: an" off the Shelf Solution" for Critical Limb Ischemia? *J. translational Med.* 6, 1–8. doi:10.1186/1479-5876-6-45

Mutirangura, P., Ruangsetakit, C., Wongwanit, C., Chinsakchai, K., Porat, Y., Belleli, A., et al. (2009). Enhancing Limb Salvage by Non-Mobilized Peripheral Blood Angiogenic Cell Precursors Therapy in Patients with Critical Limb Ischemia. *J. Med. Assoc. Thai* 92, 320–327.

Narula, N., Dannenberg, A. J., Olin, J. W., Bhatt, D. L., Johnson, K. W., Nadkarni, G., et al. (2018). Pathology of Peripheral Artery Disease in Patients with Critical Limb Ischemia. *J. Am. Coll. Cardiol.* 72, 2152–2163. doi:10.1016/j.jacc.2018.08.002

Navaei-Nigjeh, M., Amoabedini, G., Noroozi, A., Azami, M., Asmani, M. N., Ebrahimi-Barough, S., et al. (2014). Enhancing Neuronal Growth from Human Endometrial Stem Cells Derived Neuron-Like Cells in Three-Dimensional Fibrin Gel for Nerve Tissue Engineering. *J. Biomed. Mater. Res. Part A* 102, 2533–2543. doi:10.1002/jbm.a.34921

Ohtake, T., Mochida, Y., Ishioka, K., Oka, M., Maesato, K., Moriya, H., et al. (2018). Autologous Granulocyte Colony-Stimulating Factor-Mobilized Peripheral Blood CD34 Positive Cell Transplantation for Hemodialysis Patients with Critical Limb Ischemia: A Prospective Phase II Clinical Trial. *Stem Cell Translational Med.* 7, 774–782. doi:10.1002/sctm.18-0104

Pan, T., Liu, H., Fang, Y., Wei, Z., Gu, S., Fang, G., et al. (2019). Predictors of Responders to Mononuclear Stem Cell-Based Therapeutic Angiogenesis for No-Option Critical Limb Ischemia. *Stem Cel. Res. Ther.* 10, 1–13. doi:10.1186/s13287-018-1117-5

Park, C.-H., Bae, S. H., Kim, H. Y., Kim, J. K., Jung, E. S., Chun, H. J., et al. (2013). A Pilot Study of Autologous CD34-Depleted Bone Marrow Mononuclear Cell Transplantation via the Hepatic Artery in Five Patients with Liver Failure. *Cytotherapy* 15, 1571–1579. doi:10.1016/j.jcyt.2013.05.013

Peplow, P. V. (2014). Growth Factor-And Cytokine-Stimulated Endothelial Progenitor Cells in Post-Ischemic Cerebral Neovascularization. *Neural Regen. Res.* 9, 1425. doi:10.4103/1673-5374.139457

Perrelli, M.-G., Pagliaro, P., and Penna, C. (2011). Ischemia/Reperfusion Injury and Cardioprotective Mechanisms: Role of Mitochondria and Reactive Oxygen Species. *World J. Cardiol.* 3, 186. doi:10.4330/wjc.v3.i6.186

Phinney, D. G., and Pittenger, M. F. (2017). Concise Review: MSC-Derived Exosomes for Cell-free Therapy. *Stem Cells* 35, 851–858. doi:10.1002/stem.2575

Piccinato, C. A., Sertie, A. L., Torres, N., Ferretti, M., and Antonioli, E. (2015). High OCT4 and Low Expressions Determine *In Vitro* Lifespan of Mesenchymal Stem Cells. *Stem Cell Int.* 2015. doi:10.1155/2015/369828

Piccirillo, N., Ausoni, G., Chiusolo, P., Sorà, F., Putzulu, R., Bianchi, M., et al. (2013). Twenty Years of Unrestricted Hematopoietic Stem Cell Collection and Storage: Impact of Joint Accreditation Committee International Society for Cellular Therapy Europe Standards Implementation on Stem Cell Storage Policy and Resource Utilization. *Cytotherapy* 15, 519–521. doi:10.1016/j.jcyt.2012.12.003

Pignon, B., Sevestre, M.-A., Kanagaratnam, L., Pernod, G., Stephan, D., Emmerich, J., et al. (2017). Autologous Bone Marrow Mononuclear Cell Implantation and its Impact on the Outcome of Patients with Critical Limb Ischemia—Results of a Randomized, Double-Blind, Placebo-Controlled Trial. *Circ. J.* 81, 1713–1720. doi:10.1253/circj.cj-17-0045

Pluchino, S., Smith, J. A., and Peruzzotti-Jametti, L. (2020). Promises and Limitations of Neural Stem Cell Therapies for Progressive Multiple Sclerosis. *Trends Mol. Med.* 26, 898–912. doi:10.1016/j.molmed.2020.04.005

Portnow, J., Synold, T. W., Badie, B., Tirughana, R., Lacey, S. F., D'apuzzo, M., et al. (2017). Neural Stem Cell–Based Anticancer Gene Therapy: A First-In-Human Study in Recurrent High-Grade Glioma Patients. *Clin. Cancer Res.* 23, 2951–2960. doi:10.1158/1078-0432.ccr-16-1518

Prianishnikov, V. (1978). On the Concept of Stem Cell and a Model of Functional-Morphological Structure of the Endometrium. *Contraception* 18, 213–223. doi:10.1016/s0010-7824(78)80015-8

Qiu, Z., Zhu, L., He, L., Chen, D., Zeng, D., Chen, G., et al. (2019). DNA Damage and Reactive Oxygen Species Cause Cell Death in the rice Local Lesions 1 Mutant Under High Light and High Temperature. *New Phytol.* 222, 349–365. doi:10.1111/nph.15597

Reali, C., Scintu, F., Pillai, R., Cabras, S., Argiolu, F., Ristaldi, M. S., et al. (2006). Differentiation of Human Adult CD34+ Stem Cells into Cells with a Neural Phenotype: Role of Astrocytes. *Exp. Neurol.* 197, 399–406. doi:10.1016/j.expneurol.2005.10.004

Rigato, M., Monami, M., and Fadini, G. P. (2017). Autologous Cell Therapy for Peripheral Arterial Disease: Systematic Review and Meta-Analysis of

Randomized, Nonrandomized, and Noncontrolled Studies. *Circ. Res.* 120, 1326–1340. doi:10.1161/circresaha.116.309045

Rink, B. E., Amilon, K. R., Esteves, C. L., French, H. M., Watson, E., Aurich, C., et al. (2017). Isolation and Characterization of Equine Endometrial Mesenchymal Stromal Cells. *Stem Cel. Res. Ther.* 8, 1–12. doi:10.1186/s13287-017-0616-0

Sarasúa, J. G., López, S. P., Viejo, M. Á., Basterrechea, M. P., Rodríguez, A. F., Gutiérrez, A. F., et al. (2011). Treatment of Pressure Ulcers with Autologous Bone Marrow Nuclear Cells in Patients with Spinal Cord Injury. *J. Spinal Cord Med.* 34, 301–307. doi:10.1179/2045772311y.0000000010

Sassoli, C., Nosi, D., Tani, A., Chellini, F., Mazzanti, B., Quercioli, F., et al. (2014). Defining the Role of Mesenchymal Stromal Cells on the Regulation of Matrix Metalloproteinases in Skeletal Muscle Cells. *Exp. Cel. Res.* 323, 297–313. doi:10.1016/j.yexcr.2014.03.003

Sasson, I. E., and Taylor, H. S. (2008). Stem Cells and the Pathogenesis of Endometriosis. *Ann. New York Acad. Sci.* 1127, 106–115. doi:10.1196/annals.1434.014

Schröder, K., Zhang, M., Benkhoff, S., Mieth, A., Pliquett, R., Kosowski, J., et al. (2012). Nox4 Is a Protective Reactive Oxygen Species Generating Vascular NADPH Oxidase. *Circ. Res.* 110, 1217–1225. doi:10.1161/circresaha.112.267054

Sedger, L. M., and Mcdermott, M. F. (2014). TNF and TNF-Receptors: From Mediators of Cell Death and Inflammation to Therapeutic Giants–Past, Present and Future. *Cytokine Growth Factor Rev.* 25, 453–472. doi:10.1016/j.cytogfr.2014.07.016

Seebach, C., Henrich, D., Meier, S., Nau, C., Bonig, H., and Marzi, I. (2016). Safety and Feasibility of Cell-Based Therapy of Autologous Bone Marrow-Derived Mononuclear Cells in Plate-Stabilized Proximal Humeral Fractures in Humans. *J. Translational Med.* 14, 1–11. doi:10.1186/s12967-016-1066-7

Seekamp, A., Warren, J. S., Remick, D., Till, G., and Ward, P. (1993). Requirements for Tumor Necrosis Factor-Alpha and Interleukin-1 in Limb Ischemia/Reperfusion Injury and Associated Lung Injury. *Am. J. Pathol.* 143, 453.

Shamosi, A., Farokhi, M., Ai, J., and Sharifi, E. (2015). Induction of Spontaneous Neo-Angiogenesis and Tube Formation in Human Endometrial Stem Cells by Bioglass. *J. Med. Hypotheses Ideas* 9, 94–98. doi:10.1016/j.jmhi.2015.09.004

Shamosi, A., Mehrabani, D., Azami, M., Ebrahimi-Barough, S., Siavashi, V., Ghanbari, H., et al. (2017). Differentiation of Human Endometrial Stem Cells into Endothelial-Like Cells on Gelatin/Chitosan/Bioglass Nanofibrous Scaffolds. *Artif. Cell Nanomedicine, Biotechnol.* 45, 163–173. doi:10.3109/21691401.2016.1138493

Shi, M., Zhang, Z., Xu, R., Lin, H., Fu, J., Zou, Z., et al. (2012). Human Mesenchymal Stem Cell Transfusion Is Safe and Improves Liver Function in Acute-on-Chronic Liver Failure Patients. *Stem Cell Translational Med.* 1, 725–731. doi:10.5966/sctm.2012-0034

Shirian, S., Ebrahimi-Barough, S., Saberi, H., Norouzi-Javidan, A., Mousavi, S. M. M., Derakhshan, M. A., et al. (2016). Comparison of Capability of Human Bone Marrow Mesenchymal Stem Cells and Endometrial Stem Cells to Differentiate into Motor Neurons on Electrospun Poly (ε-Caprolactone) Scaffold. *Mol. Neurobiol.* 53, 5278–5287. doi:10.1007/s12035-015-9442-5

Shoji, K., Zen, K., Yanishi, K., Wakana, N., Nakanishi, N., Nakamura, T., et al. (2020). Two Effective Cases of Additional Pedal Artery Angioplasty for Severe Lower Limb Ischemia Following Acute Thrombotic Artery Occlusion with Hypercoagulable State Diseases. *CVIR Endovascular* 3, 1–5. doi:10.1186/s42155-020-00166-7

Simon, F., Oberhuber, A., Floros, N., Busch, A., Wagenhäuser, M. U., Schelzig, H., et al. (2018). Acute Limb Ischemia—Much More Than Just a Lack of Oxygen. *Int. J. Mol. Sci.* 19, 374. doi:10.3390/ijms19020374

Singer, W., Dietz, A. B., Zeller, A. D., Gehrking, T. L., Schmelzer, J. D., Schmeichel, A. M., et al. (2019). Intrathecal Administration of Autologous Mesenchymal Stem Cells in Multiple System Atrophy. *Neurology* 93, e77–e87. doi:10.1212/wnl.0000000000007720

Steiling, H., Munz, B., Werner, S., and Brauchle, M. (1999). Different Types of ROS-Scavenging Enzymes Are Expressed During Cutaneous Wound Repair. *Exp. Cel. Res.* 247, 484–494. doi:10.1006/excr.1998.4366

Stein, J., Steven, S., Bros, M., Sudowe, S., Hausding, M., Oelze, M., et al. (2017). Role of Protein Kinase C and Nox2-Derived Reactive Oxygen Species Formation in the Activation and Maturation of Dendritic Cells by Phorbol Ester and Lipopolysaccharide. *Oxidative Med. Cell. Longevity* 2017, 4157213. doi:10.1155/2017/4157213

Sullivan, R., Dailey, T., Duncan, K., Abel, N., and Borlongan, C. V. (2016). Peripheral Nerve Injury: Stem Cell Therapy and Peripheral Nerve Transfer. *Int. J. Mol. Sci.* 17, 2101. doi:10.3390/ijms17122101

Tabatabaei, F. S., Ai, J., Kashi, T. S. J., Khazaei, M., Kajbafzadeh, A.-M., and Ghanbari, Z. (2013). Effect of Dentine Matrix Proteins on Human Endometrial Adult Stem-Like Cells: *In Vitro* Regeneration of Odontoblasts Cells. *Arch. Oral Biol.* 58, 871–879. doi:10.1016/j.archoralbio.2013.01.013

Taghdiri Nooshabadi, V., Verdi, J., Ebrahimi-Barough, S., Mowla, J., Atlasi, M. A., Mazoochi, T., et al. (2019). Endometrial Mesenchymal Stem Cell-Derived Exosome Promote Endothelial Cell Angiogenesis in a Dose Dependent Manner: A New Perspective on Regenerative Medicine and Cell-Free Therapy. *Arch. Neurosci.* 6. doi:10.5812/ans.94041

Tanaka, R., Masuda, H., Kato, S., Imagawa, K., Kanabuchi, K., Nakashioya, C., et al. (2014). Autologous G-CSF-Mobilized Peripheral Blood CD34+ Cell Therapy for Diabetic Patients with Chronic Nonhealing Ulcer. *Cel. Transplant.* 23, 167–179. doi:10.3727/096368912x658007

Tanniou, J., Van Der Tweel, I., Teerenstra, S., and Roes, K. C. (2016). Subgroup Analyses in Confirmatory Clinical Trials: Time to Be Specific About Their Purposes. *BMC Med. Res. Methodol.* 16, 1–15. doi:10.1186/s12874-016-0122-6

Tao, H., Han, Z., Han, Z. C., and Li, Z. (2016). Proangiogenic Features of Mesenchymal Stem Cells and Their Therapeutic Applications. *Stem Cell Int.* 2016. doi:10.1155/2016/1314709

Tateishi-Yuyama, E., Matsubara, H., Murohara, T., Ikeda, U., Shintani, S., Masaki, H., et al. (2002). Therapeutic Angiogenesis for Patients with Limb Ischaemia by Autologous Transplantation of Bone-Marrow Cells: A Pilot Study and a Randomised Controlled Trial. *The Lancet* 360, 427–435. doi:10.1016/s0140-6736(02)09670-8

Tehrani, H. J., Parivar, K., Ai, J., Kajbafzadeh, A., Rahbarghazi, R., Hashemi, M., et al. (2014). Effect of Dexamethasone, Insulin and EGF on the Myogenic Potential on Human Endometrial Stem Cell. *Iranian J. Pharm. Res. IJPR* 13, 659.

Tendera, M., Wojakowski, W., Rużyłło, W., Chojnowska, L., Kępka, C., Tracz, W., et al. (2009). Intracoronary Infusion of Bone Marrow-Derived Selected CD34+ CXCR4+ Cells and Non-Selected Mononuclear Cells in Patients with Acute STEMI and Reduced Left Ventricular Ejection Fraction: Results of Randomized, Multicentre Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction (REGENT) Trial. *Eur. Heart J.* 30, 1313–1321. doi:10.1093/eurheartj/ehp073

Torbjörnsson, E., Fagerdahl, A.-M., Blomgren, L., Boström, L., Ottosson, C., and Malmstedt, J. (2021). Risk Factors for Reamputations in Patients Amputated after Revascularization for Critical Limb-Threatening Ischemia. *J. Vasc. Surg.* 73, 258–266. e251. doi:10.1016/j.jvs.2020.03.055

Toyoda, M., Cui, C., and Umezawa, A. (2007). Myogenic Transdifferentiation of Menstrual Blood-Derived Cells. *Acta Myologica* 26, 176.

Uccioli, L., Meloni, M., Izzo, V., Giurato, L., Merolla, S., and Gandini, R. (2018). Critical Limb Ischemia: Current Challenges and Future Prospects. *Vasc. Health Risk Manag.* 14, 63. doi:10.2147/vhrm.s125065

Urbich, C., and Dimmeler, S. (2004). Endothelial Progenitor Cells: Characterization and Role in Vascular Biology. *Circ. Res.* 95, 343–353. doi:10.1161/01.res.0000137877.89448.78

Verdi, J., Tan, A., Shoae-Hassani, A., and Seifalian, A. M. (2014). Endometrial Stem Cells in Regenerative Medicine. *J. Biol. Eng.* 8, 1–10. doi:10.1186/1754-1611-8-20

Viganò, M., Budelli, S., Lavazza, C., Montemurro, T., Montelatici, E., De Cesare, S., et al. (2018). Tips and Tricks for Validation of Quality Control Analytical Methods in Good Manufacturing Practice Mesenchymal Stromal Cell Production. *Stem Cell Int.* 2018. doi:10.1155/2018/3038565

Wahid, F. S. A., Ismail, N. A., Wan Jamaludin, W. F., Muhamad, N. A., Mohamad Idris, M. A., and Lai, N. M. (2018). Efficacy and Safety of Autologous Cell-Based Therapy in Patients with No-Option Critical Limb Ischaemia: A Meta-Analysis. *Curr. Stem Cel. Res. Ther.* 13, 265–283. doi:10.2174/1574888x13666180313141416

Wang, Q., Guerrero, F., Mazur, A., Lambrechts, K., Buzzacott, P., Belhomme, M., et al. (2015). Reactive Oxygen Species, Mitochondria, and Endothelial Cell Death During *In Vitro* Simulated Dives. *Med. Sci. Sports Exerc.* 47, 1362–1371. doi:10.1249/mss.0000000000000563

Wang, S. K., Green, L. A., Drucker, N. A., Motaganahalli, R. L., Fajardo, A., and Murphy, M. P. (2018). Rationale and Design of the Clinical and Histologic

Analysis of Mesenchymal Stromal Cells in AmPutations (CHAMP) Trial Investigating the Therapeutic Mechanism of Mesenchymal Stromal Cells in the Treatment of Critical Limb Ischemia. *J. Vasc. Surg.* 68, 176–181. e171. doi:10.1016/j.jvs.2017.09.057

Weem, S. P., Teraa, M., Den Ruijter, H., De Borst, G., Verhaar, M., and Moll, F. (2016). Quality of Life After Treatment with Autologous Bone Marrow Derived Cells in No Option Severe Limb Ischemia. *Eur. J. Vasc. Endovascular Surg.* 51, 83–89. doi:10.1016/j.ejvs.2015.09.010

Wijnand, J. G., Teraa, M., Gremmels, H., Van Rhijn-Brouwer, F. C., De Borst, G. J., Verhaar, M. C., et al. (2018). Rationale and Design of the SAIL Trial for Intramuscular Injection of Allogeneic Mesenchymal Stromal Cells in No-Option Critical Limb Ischemia. *J. Vasc. Surg.* 67, 656–661. doi:10.1016/j.jvs.2017.09.026

Wilson, J. G., Liu, K. D., Zhuo, H., Caballero, L., Mcmillan, M., Fang, X., et al. (2015). Mesenchymal Stem (Stromal) Cells for Treatment of ARDS: A Phase 1 Clinical Trial. *Lancet Respir. Med.* 3, 24–32. doi:10.1016/s2213-2600(14)70291-7

Wu, Z., Cai, J., Chen, J., Huang, L., Wu, W., Luo, F., et al. (2014). Autologous Bone Marrow Mononuclear Cell Infusion and Hyperbaric Oxygen Therapy in Type 2 Diabetes Mellitus: An Open-Label, Randomized Controlled Clinical Trial. *Cytotherapy* 16, 258–265. doi:10.1016/j.jcyt.2013.10.004

Xia, C., Zeng, Z., Fang, B., Tao, M., Gu, C., Zheng, L., et al. (2019). Mesenchymal Stem Cell-Derived Exosomes Ameliorate Intervertebral Disc Degeneration via Anti-Oxidant and Anti-Inflammatory Effects. *Free Radic. Biol. Med.* 143, 1–15. doi:10.1016/j.freeradbiomed.2019.07.026

Xiao, Z., Tang, F., Zhao, Y., Han, G., Yin, N., Li, X., et al. (2018). Significant Improvement of Acute Complete Spinal Cord Injury Patients Diagnosed by a Combined Criteria Implanted with Neuroregen Scaffolds and Mesenchymal Stem Cells. *Cel. Transplant.* 27, 907–915. doi:10.1177/0963689718766279

Xu, B., Fan, D., Zhao, Y., Li, J., Wang, Z., Wang, J., et al. (2020). Three-Dimensional Culture Promotes the Differentiation of Human Dental Pulp Mesenchymal Stem Cells into Insulin-Producing Cells for Improving the Diabetes Therapy. *Front. Pharmacol.* 10, 1576. doi:10.3389/fphar.2019.01576

Yamamoto, K., Kondo, T., Suzuki, S., Izawa, H., Kobayashi, M., Emi, N., et al. (2004). Molecular Evaluation of Endothelial Progenitor Cells in Patients with Ischemic Limbs: Therapeutic Effect by Stem Cell Transplantation. *Arteriosclerosis, Thromb. Vasc. Biol.* 24, E192–E196. doi:10.1161/01.ATV.0000147730.78953.60

Yañez, R., Lamana, M. L., García-Castro, J., Colmenero, I., Ramírez, M., and Bueren, J. A. (2006). Adipose Tissue-Derived Mesenchymal Stem Cells Have *In Vivo* Immunosuppressive Properties Applicable for the Control of the Graft-Versus-Host Disease. *Stem Cells* 24, 2582–2591. doi:10.1634/stemcells.2006-0228

Ye, J., and Yuan, L. (2007). Inhibition of P38 MAPK Reduces Tumor Conditioned Medium-Induced Angiogenesis in Co-cultured Human Umbilical Vein Endothelial Cells and Fibroblasts. *Biosci. Biotechnol. Biochem.* 71, 1162–1169. doi:10.1271/bbb.60617

Yoon, Y., Kim, H. S., Jeon, I., Noh, J. E., Park, H. J., Lee, S., et al. (2020). Implantation of the Clinical-Grade Human Neural Stem Cell Line, CTX0E03, Rescues the Behavioral and Pathological Deficits in the Quinolinic Acid-Lesioned Rodent Model of Huntington's Disease. *Stem Cells* 38, 936–947. doi:10.1002/stem.3191

Yu, J., and Dardik, A. (2018). A Murine Model of Hind Limb Ischemia to Study Angiogenesis and Arteriogenesis. *Traumatic Ischemic Inj.*, 135–143. doi:10.1007/978-1-4939-7526-6_11

Zabel, M., Willems, R., Lubinski, A., Bauer, A., Brugada, J., Conen, D., et al. (2020). Clinical Effectiveness of Primary Prevention Implantable Cardioverter-Defibrillators: Results of the EU-CERT-ICD Controlled Multicentre Cohort Study. *Eur. Heart J.* 41, 3437–3447. doi:10.1093/eurheartj/ehaa226

Zafarghandi, M. R., Ravari, H., Aghdami, N., Namiri, M., Moazzami, K., Taghiabadi, E., et al. (2010). Safety and Efficacy of Granulocyte–Colony-Stimulating Factor Administration Following Autologous Intramuscular Implantation of Bone Marrow Mononuclear Cells: A Randomized Controlled Trial in Patients with Advanced Lower Limb Ischemia. *Cytotherapy* 12, 783–791. doi:10.3109/14653240903518163

Zannettino, A., Paton, S., Arthur, A., Khor, F., Itescu, S., Gimble, J., et al. (2008). Multipotential Human Adipose-Derived Stromal Stem Cells Exhibit a Perivascular Phenotype *In Vitro* and *In Vivo*. *J. Cell. Physiol.* 214, 413–421. doi:10.1002/jcp.21210

Zhong, Z., Patel, A. N., Ichim, T. E., Riordan, N. H., Wang, H., Min, W.-P., et al. (2009). Feasibility Investigation of Allogeneic Endometrial Regenerative Cells. *J. Translational Med.* 7, 1–7. doi:10.1186/1479-5876-7-15

Zhou, S.-B., Zhang, G.-Y., Xie, Y., Zan, T., Gan, Y.-K., Yao, C. A., et al. (2016). Autologous Stem Cell Transplantation Promotes Mechanical Stretch Induced Skin Regeneration: A Randomized Phase I/II Clinical Trial. *EBioMedicine* 13, 356–364. doi:10.1016/j.ebiom.2016.09.031

Zhu, J., Song, J., Yu, L., Zheng, H., Zhou, B., Weng, S., et al. (2016). Safety and Efficacy of Autologous Thymosin β4 Pre-Treated Endothelial Progenitor Cell Transplantation in Patients with Acute ST Segment Elevation Myocardial Infarction: A Pilot Study. *Cytotherapy* 18, 1037–1042. doi:10.1016/j.jcyt.2016.05.006

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# Single-Cell Transcriptomics Uncover Key Regulators of Skin Regeneration in Human Long-Term Mechanical Stretch-Mediated Expansion Therapy

*Yidan Sun[1†], Luwen Xu[1†], Yin Li[2†], Jian Lin[3], Haizhou Li[1], Yashan Gao[1], Xiaolu Huang[1], Hainan Zhu[1], Yingfan Zhang[1], Kunchen Wei[1], Yali Yang[4,5], Baojin Wu[6], Liang Zhang[2,7]*, Qingfeng Li[1]* and Caiyue Liu[1]**

[1]Department of Plastic and Reconstructive Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2]CAS Key Laboratory of Tissue Microenvironment and Tumor, Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai, China, [3]Department of Orthopedics, Shanghai Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [4]Department of Dermatology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [5]Department of Laser Cosmetology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [6]Department of Plastic Surgery, Shanghai Huashan Hospital, Fudan University School of Medicine, Shanghai, China, [7]Institute for Stem Cell and Regeneration, Chinese Academy of Sciences, Beijing, China

Tissue expansion is a commonly performed therapy to grow extra skin *in vivo* for reconstruction. While mechanical stretch-induced epidermal changes have been extensively studied in rodents and cell culture, little is known about the mechanobiology of the human epidermis *in vivo*. Here, we employed single-cell RNA sequencing to interrogate the changes in the human epidermis during long-term tissue expansion therapy in clinical settings. We also verified the main findings at the protein level by immunofluorescence analysis of independent clinical samples. Our data show that the expanding human skin epidermis maintained a cellular composition and lineage trajectory that are similar to its non-expanding neighbor, suggesting the cellular heterogeneity of long-term expanded samples differs from the early response to the expansion. Also, a decrease in proliferative cells due to the decayed regenerative competency was detected. On the other hand, profound transcriptional changes are detected for epidermal stem cells in the expanding skin versus their non-expanding peers. These include significantly enriched signatures of C-FOS, EMT, and mTOR pathways and upregulation of AREG and SERPINB2 genes. CellChat associated ligand-receptor pairs and signaling pathways were revealed. Together, our data present a single-cell atlas of human epidermal changes in long-term tissue expansion therapy, suggesting that transcriptional change in epidermal stem cells is the major mechanism underlying long-term human skin expansion therapy. We also identified novel therapeutic targets to promote human skin expansion efficiency in the future.

**Keywords: skin regeneration, mechanical stretch, tissue expansion, single cell RNA sequence, epithelial to mesenchymal transformation, c-fos, AREG–EREG ligands**

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*Kun Yang, East Tennessee State University, United States*

***Correspondence:**
*Liang Zhang*
*zhangliang01@sibs.ac.cn*
*Qingfeng Li*
*dr.liqingfeng@shsmu.edu.cn*
*Caiyue Liu*
*yueyue1888@my.com;*
*yueyue1888@hotmail.com*

*[†]These authors have contributed equally to this work and share first authorship*

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## INTRODUCTION

As the outmost barrier of our body, skin has a high demand for renewal and regeneration during physiological growth stimuli and following injury (Biggs et al., 2020). Mechanical stretch-mediated tissue expansion is the epitome of capitalizing on skin's mechanobiological characterization to repair defects and scar reconstruction in plastic and reconstructive surgery (Zöllner et al., 2013). Ordinary routine expansion includes procedures such as surgically inserting expanders beneath the skin, weekly filling expanders by serial injection of sterile isotonic saline solution over a period, and gradually generating extra skin *in situ* (Huang et al., 2011; Zhu et al., 2018; Topczewska et al., 2019). Thus, we demonstrated that human skin expansion therapy follows a moderate constant volume skin expansion paradigm (MCVSE), in which a moderate stretching force is applied progressively and the skin is maintained in an expanded stage throughout the whole treatment process. It is a very slow process to induce skin regeneration that usually takes at least 8–12 weeks to achieve 3-fold skin growth, but this gradual expansion permits better tissue tolerance. Notably, the clinical expansion is usually capped at about 4-fold while parts of the skin become extremely thin, implicating exhaustion of skin regeneration competency during long-term expansion (LTE) therapy (Li et al., 2016; Huang et al., 2021, 1; Tepole et al., 2012).

Additionally, the rapid skin expansion process (RSE) based on a self-inflating tissue expander is also used in clinical therapy to avoid multiple injections and reduce expansion-related discomfort (Obdeijn et al., 2009). However, once the expansion has begun, there is no possibility to adjust the expansion speed, and difficulties are presented if there are any problems with the overlying skin viability (Lohana et al., 2012). Therefore, choosing between slow or rapid expansion, and small or large inflation volumes, remains controversial, and gold-standard procedures for reconstructive surgery are still under debate (Lee et al., 2018). Recently, a seminal study (Aragona et al., 2020) focusing on the RSE process employed a subcutaneous rapid inflating hydrogel mouse model to explore the mechanism of stretch-mediated skin expansion *in vivo*. Their findings include that stretch induces major changes in epidermal lineage trajectory, including the emergence of a stem cell-like "stretch" cell population that is distinct from regular epidermal basal cells and tips the renewal-differentiation balance to accelerate the production of more stem cells. Also, stretch changed stem cells at the molecular level involved in cell-cell adhesion, actomyosin cytoskeleton, and induced a network of regulatory genes, such as the activation of MEK-ERK–AP1 and YAP-MAL signaling. However, we assume that the mechanistic study of skin therapy based on the RSE model is not enough to closely mimic the changes in human skin in MCVSE clinical settings. Further characterization of the relevance of the main findings from the RSE model to the clinical MCVSE process was warranted. Moreover, exactly how human long-term expansion therapy changes skin phenotypes at the single-cell level in a living organism has not been known, and a better description of LTE would be of absolute importance to the field.

In this study, after a combination of single-cell RNA sequencing (scRNAseq) analysis and two paired long-term expanded skin samples and immunofluorescent (IF) staining of more samples from four individuals from surgical discards, we found that although epithelial lineage identity remains unvaried, numbers of proliferative epithelial cells after long-term expansion decline. Furthermore, they did exhibit prevalent transcriptional changes, particularly in C-FOS, EMT, and mTOR networks, and these were accompanied by skin's adaption and balance capacity to maintain homeostasis under LTE. Moreover, marked expansion-upregulated genes including AREG and SERPINB2 were found. We also identified that LTE triggered three common pairs of ligand-receptor networks including CD96-NECTIN1, AREG-EGFR, and LAMININ-CD44, and three signaling pathways, including EGF, LAMININ, and NECTIN based on CellChat analysis. Overall, our data present a single-cell atlas of human epidermal changes in long-term tissue expansion therapy, suggesting that transcriptional change in epidermal stem cells is the major mechanism underlying human long-term skin expansion therapy. Our findings also revealed promising therapeutic targets for promoting skin regeneration clinically in the future.

## METHODS

### Ethics Statement

The humans expanded and their nearby samples were obtained from discarded plastic surgery specimens in Shanghai's 9th People's Hospital (Shanghai, China). Individuals with more than 5 months of the expanded duration were enrolled in the study ($n$ = 6). Specifically, samples used for transcriptomic analysis were from two female donors and were collected from different skin areas. They experienced full facial reconstruction with total autologous tissue transplantation and 10-month saline injection. Informed consent from all patients and/or guardians was signed before sample collection in accordance with the Declaration of Helsinki and with approval from the Human Research Ethics Committee of Shanghai Jiao Tong University School of Medicine (Shanghai, China). Detailed patient information is summarized in **Table 1**.

### Tissue Dissociation and Cell Isolation

Skin samples were collected and stored in ice-cold phosphate buffer saline (PBS, Sigma-Aldrich) after surgery. For epidermis isolation, subcutaneous tissues were removed from samples and the epidermis was enzymatically dissociated from the dermis with dispase digestion (90% DMEM, 10% Fetal Bovine Serum, 2 mg/ml dispase II, 1% penicillin-streptomycin solution) incubation at 37°C overnight. Epidermal sheets were then manually separated from the dermis and then dissociated into single cells with trypsin-versene (Lonza) incubation at 115 rpm for 10 min at 37°C. The generated single-cell suspensions in 50 ml of 0.04% bovine serum albumin (BSA, Gibco) in PBS were used for ×10 Genomic sequencing.

**TABLE 1 |** Clinical information of patients whose samples were used in this study.

| Sex | Age (y) | Site | Race | Expanded duration (m) | scRNA-seq analysis | IF staining | Abbreviations |
|---|---|---|---|---|---|---|---|
| Female | 19 | Face | Han nationality | 10 | Yes | | F-Exp and F-Nby |
| Female | 23 | Neck | Han nationality | 10 | Yes | | N-Exp and N-Nby |
| Male | 18 | Head | Han nationality | 9 | | Yes | M18 H9 |
| Female | 25 | Back | Han nationality | 11 | | Yes | F25 B11 |
| Male | 16 | Back | Han nationality | 13 | | Yes | M16 B13 |
| Female | 17 | Neck | Han nationality | 14 | | Yes | F17 N14 |

## Single-Cell RNA Sequencing

The cell suspense dissociated above was loaded into ×10 Chromium controller to generate GEMs with gel beads. The GEMs first reversed transcript to cDNA and then further processed into single-cell 3′ gene expression libraries according to the manufacturers' instruction manual. In short, the GEMs were reverse transcribed to ss-cDNA first. The single-strand cDNA was purified by beads and amplified by PCR to generate the ds-cDNA. Next, ds-cDNA was fragmented, end-repaired, and further ligated with an adaptor. Lastly, index PCR was performed before sequencing. Sequencing was performed on the Illumina Nova-seq platform.

## Single-Cell Transcriptomic Analysis

Raw FASTQ files were first trimmed by TrimGalore with the parameter "-q 30–phred33–stringency 3–length 20-e 0.1". Clean FASTQ was processed by the Cell Ranger (v4.0.0) pipeline. After obtaining the UMI matrix, Seurat (v4.0.5) was used for filtering and preprocessing the data. Quality control was performed using the subset function using the threshold of nFeature_RNA larger than 800 and less than 10,000, as well as the percentage of expressed mitochondrial genes less than 20% to filter out low-quality cells and potential doublets. To visualize the data in UMAP and clustering cells, we set the final resolution to 0.5 (testing a range from 0.2–1.0) and dims to 34 (testing a range from 10–50).

## Data Integration

To compare the single-cell RNA-seq data from the adjacent expansion and nearby skin samples, data integration was performed using the CCA algorithm. 2,400 features were selected to find anchors between samples. Further downstream analyses such as dimensionality reduction and clustering were all performed as described.

## Trajectory Analysis

The pseudotime analysis was done by Monocle3 (Cao et al., 2019) and scVelo (Bergen et al., 2020) to reconstruct the epidermal cell developmental trajectory. According to prior knowledge of differentiation from basal cells to spinous cells, the "root" cell is chosen at the very beginning of basal cell in Monocle3. The IFE cells were further divided into three stages according to the pseudotime and defined as "EDC", "MDC," and "LDC". To map the differentiation trajectory directions, scVelo was used to calculate the RNA velocity. The cell filter mentioned above was used to calculate the transcriptional dynamics of splicing kinetics. The standard dynamical modeling workflow was used to obtain the stream plot of velocities.

## Gene Ontology Analysis

GO analysis of DEGs was performed by Metascape (version 3.5, http://metascape.org/) and visualized with the ggplot2 R package (https://github.com/tidyverse/ggplot2) and prism 9. Representative terms of biological processes (BP) selected from the top 20 ranked GO terms or pathways ($p < 0.01$) were displayed.

## Gene Set Enrichment Analysis

The gene set enrichment analysis was performed using the single seqgset R package (https://arc85.github.io/singleseqgset/), which uses a simple underlying statistic (variance inflated Wilcoxon rank-sum testing) to determine the enrichment of gene sets of interest across clusters. The 50 hallmark genesets are downloaded from MSigDB Collections. Other self-defined genesets were listed in **Supplementary Table S1**.

## Cell-Cell Communications

To infer the intercellular communication network between clusters, the CellChat R package was used to quantitatively measure networks through the law of mass action based on the average expression values of a ligand by one cell group and that of a receptor by another cell group, as well as their cofactors. Significant interactions are identified on the basis of a statistical test that randomly permutes the group labels of cells and then recalculates the interaction probability. We also compared the cell–cell communication probability between the Exp and Nby samples. The DEGs calculated by the *identifyOverExpressedGenes* function were mapped onto inferred cell-cell communications to subset the significantly changed ligand-receptor pairs. Furthermore the upregulated pairs were shown in the bubble plot.

## Immunofluorescence Staining

For IF staining of sections, cryosections were made from frozen tissues embedded in OCT compound (Tissue Tek). The paired tissues were prepared on the same slide to ensure the same staining conditions. Slides were fixed for 10 min in 4% paraformaldehyde and blocked for 1 h in blocking buffer (2.5% normal donkey serum+2.5% normal goat serum+1% BSA+0.3% Triton X-100). Sections were then incubated with primary antibodies at 4°C overnight and with fluorochrome-conjugated secondary antibodies at room temperature for 1 h.

Slides were then washed in PBS and mounted with Fluoromount-G mounting media (Invitrogen). Images were taken by Zeiss LSM 880 upright confocal multiphoton microscope. The following antibodies dilutions were used: KRT14 (chicken, Biolegend, 1: 500), KRT15(mouse, Santa Cruz, 1:500), KRT10 (rabbit, Abcam, 1:500), KI67 (rabbit, Abcam, 1:500), PCNA(mouse, Servicebio, 1: 200), BCL-2 (mouse, Biolegend, 1:200), CD45 (mouse, Biolegend, 1:200), E-CAD (rat, eBioscience, 1:200), Phospho-mTOR (Ser2448) (rabbit, CST, 1:50), FOS (rabbit, Abcam, 1:200), P63 (rabbit, Abcam, 1:200), AREG (rabbit, Abcam, 1:200), SERPINB2 (mouse, Novusbio, 1:200), NECTIN1(rabbit, Thermofisher, 1: 100), CD96 (mouse, Santa Cruz, 1:50).

## Immunofluorescence Intensity Measurement

All photographs were taken at the same exposure time. To quantify the intensity of the immunostaining for E-CAD at the adherens junctions, we used the pseudo-color Fire from ImageJ, a well-established method of measuring fluorescence intensity that accounts for differences in the area of the signal (Ellis et al., 2019; Aragona et al., 2020), and the integrated density signal was shown in the figures.

## Statistical Analysis

For quantification of the thickness of the epidermis and papillary dermis (PD), we used the total area divided by the length of the basal membrane to get the average thickness. For quantification of the staining intensity, the expanded and their nearby counterparts were cut and placed on the same slice. They were photographed with the same exposure duration and light intensity on the same microscope mentioned before. All the results were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). Comparisons across multiple groups were made using a two-way ANOVA with the Sidak's multiple comparisons post hoc test. Differences were regarded as significant at $p < 0.05$. The quantitative data shown is expressed as mean ± standard error of the mean (SEM, represented as error bars). Graphpad Prism 9 software was used to assess statistical significance. The statistical significance level was set at $p < 0.05$.

# RESULTS

## scRNA-Seq Analysis Revealed Similar Cell Type Compositions Under Long-Term Expansion at Single-Cell Resolution

To date, seminal studies have focused on the instantaneous responses of mechanical stretch (Le et al., 2016; Aragona et al., 2020; Nava et al., 2020). Although previous research verified that skin regenerative regulation induced by mechanical stretch was time-correlated and 5-month-expansion was defined as LTE (Wang et al., 2021, 2), a detailed analysis of the LTE phenotypes of humans under clinical settings is still needed. To determine the changes in human skin epidermis during long-term (>5 months) tissue expansion therapy, we collected samples of human facial (F) and neck (N) LTE skin, respectively. Each contained paired samples from the expansion (Exp) and nearby non-expansion area (Nby) (**Table 1**). The epidermis was isolated by dispase digestion from these samples and subjected to 10x Genomics single-cell RNA-sequencing (scRNAseq) (**Figure 1A**). After stringent cell filtration, 22,223 cells were retained for subsequent analyses. We visualized human epidermal cell populations using uniform manifold approximation and projection (UMAP) (**Figure 1B**) and identified 8 major cell clusters based on the expression of classic skin lineage markers, including basal cell (BAS, *KRT14*+, *KRT15*+, *COL17A1*+), spinous cell (SPN, *KRT10*+, *KRT1*+), granular cell (GRN, *FLG*+, *IVL*+), proliferative cell (PC, *KI67*+), hair follicle (HF, $CD59^{+}$, *CD200*+), melanocyte (ME, *DCT*+, *MLANA*+), Langerhans cell (LC, *CD207*+), and T cell (TC, *CD3D*+) (**Figures 1C,D**). Besides melanocytes and immune cells, the epidermal compartment contains a clear basal cell group (BAS) that is *KRT14*+*KRT15*+*KRT10*-, a proliferative cell group (PC), several differentiated cell groups, and some intermediate cells between BAS and differentiated cells. Despite the lower number of cells, the individual analysis of each sample generated a similar distribution of clusters and identified the same major cell types, which means there is no distinct "stretch" cell group found in the Exp samples (**Figures 1E,F**). Overall, no obvious changes in the cellular compositions were detected in Exp samples versus their Nby counterparts.

## Epidermal Cells Showed No Overt Signs of Lineage Trajectory Alternation After Long-Term Expansion

Stretch-mediated skin expansion is observed as a temporary increase in stem-cell division and eventually fuels the stem-cell differentiation as extra skin is generated. Furthermore, there exists a "stem cell-like stretch cluster" as a fast-responding population of the basal stem cell (Aragona et al., 2020). To analyze the epidermal differentiation dynamics and lineage trajectory in long-term expanded epidermis, we performed pseudotime analysis using Monocle3, in which an epidermal lineage trajectory that starts with BAS cells (Early Differentiated Cluster, EDC), passes through the intermediate cells (Middle Differentiated Cluster, MDC) and ends in differentiated cells (Late Differentiated Cluster, LDC) (**Figure 2A**). This prediction was coincident with the classic model for epidermal differentiation by previous scRNA-seq studies of human skin (Cheng et al., 2018). Meanwhile, we performed RNA velocity analysis to predict the potential directionality and transitional state of the epidermal cells (**Figure 2B**). We can see a branch trajectory with two major branches in EDC, which develop into both PC and MDC. A small fraction of the upper LDC displayed the velocity vectors pointing toward PC as the terminal, indicating that both EDC and upper LDC are capable of proliferation. Furthermore, the trajectory of LDC involves the development of both committed cells and terminally differentiated cells. These findings were consistent with the classic model of epithelial self-renewal and

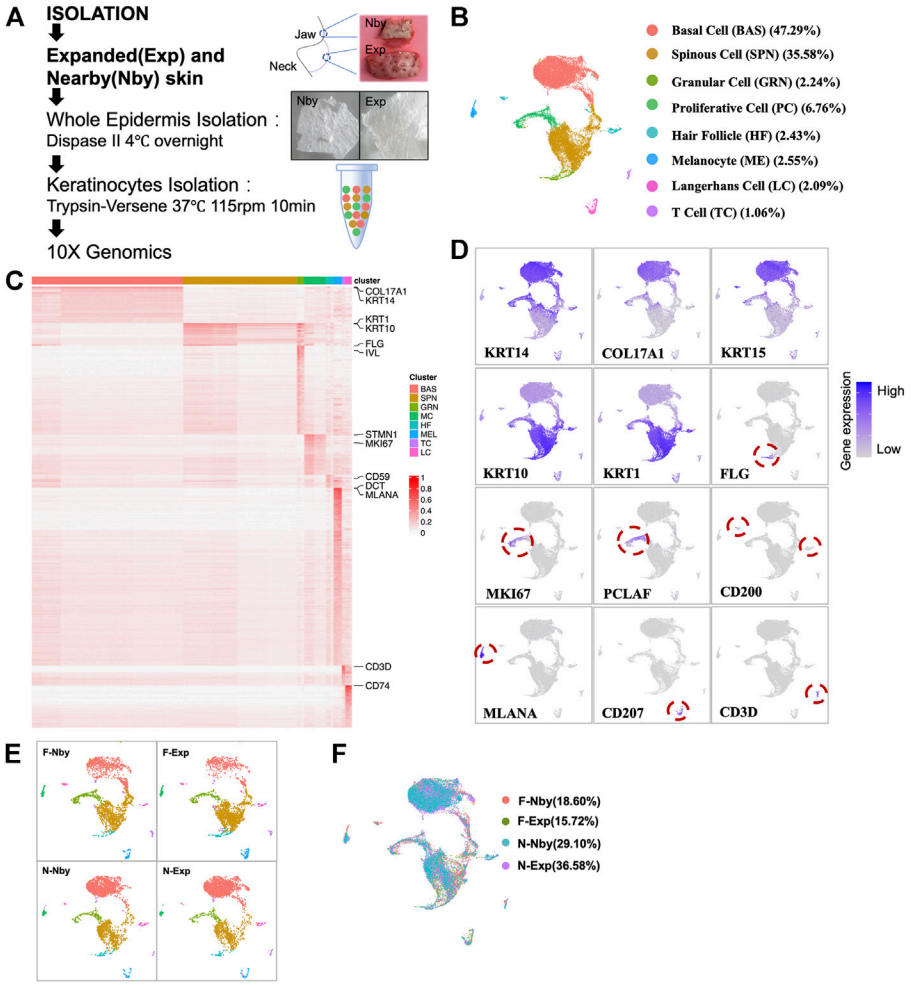


**FIGURE 1 |** ScRNA-seq reveals similar cell type composition in epidermal keratinocytes after long-term stretch. **(A)** Overview of the experimental workflow. Paradigm of single-cell isolation and sequencing strategy (right). White field images of the expanded and nearby skin of humans (left). **(B)** Uniform manifold approximation and projection (UMAP) cluster of epidermal cells ($n$ = 22223) from exp and its nby skin. Major cell types are classified using marker genes and colors corresponding to cell identity. **(C)** Heatmap of differentially expressed genes. Selected genes for each cluster are shown on the right. **(D)** UMAP plots show the representative marker genes of each cell type in human skin. The color key from gray to purple indicates low to high gene expression levels. **(E)** UMAP plot presents 3,000 cells and the same cell types in each of the four samples. Cells are colored by sample, and the percentage of each sample is annotated to the right. **(F)** UMAP plot showing similar patterns of cell clustering for each sample.

differentiated trajectory (Fuchs, 2008) but highlighted the importance of PC in skin homeostasis under stretch conditions. In contrast to the nby epidermis, cells in expanded groups identified by the differentiated stage showed inconsistent changes in numbers (**Figure 2C**). Thus, we concluded that long-term expanded human skin epidermis maintained a normal lineage trajectory. Besides, in adult primate skin, it takes ~4 weeks for a committed epidermal cell to exit the basal layer and be sloughed from the skin surface (Gonzales and Fuchs, 2017). It has "committed progenitors" belonging to basal daughters but expressing dual-differentiated markers. Researchers referred to the dually positive KRT14$^+$KRT10$^+$ cells as differentiated-poised state cells with asymmetric fate outcomes (Asare et al., 2017). The short-term expansion is always accompanied by the thickening of the epidermis, suggesting the activation of the differentiated process of the basal stem cells (Zöllner et al., 2013; Huang et al., 2021, 1). Considering KRT14$^+$KRT10$^+$ cells are the committed cells poised for differentiation, we aim to gain insight into how the differentiation tendency of the epidermal is controlled under LTE at the single-cell level. So, we observed the committed populations based on the transcriptional expression of *KRT14* and *KRT10*. Relative enrichment of committed cells (*Krt14*$^+$*Krt10*$^+$) was not observed in Exp vs. Nby comparisons for both sample pairs (**Figure 2D**). Further double staining of KRT14 and KRT10 also showed the comparable abundance of *KRT14+KRT10+* cells in Exp vs. Nby skin (**Figure 2E**). Together, these data indicate that the epidermal lineage trajectory is not significantly altered and maintains homeostasis behavior in LTE human skin under clinical conditions.

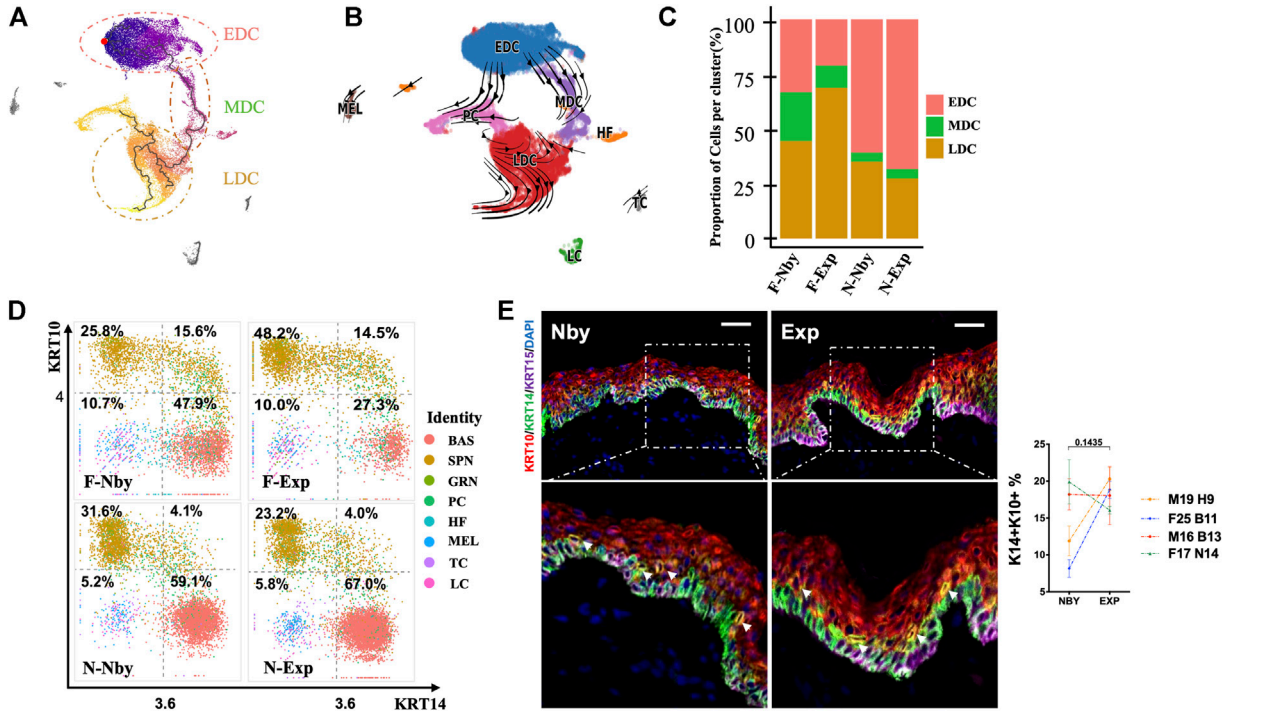


**FIGURE 2 |** Epidermis maintained a similar lineage trajectory to its counterparts after LTE. **(A)** Monocle3 pseudotemporal visualization of all the epidermal cell types along differentiation trajectories from dark purple to light yellow. The root cell was indicated in red, and the edges are shown as black line segments. Clusters were defined from early to late according to the rank of pseudotemporal cell ordering. **(B)** Velocities derived from the dynamical model for epidermal differentiated trajectory are visualized as streamlines in a UMAP-based embedding. Cells are color-coded by clusters. **(C)** The fraction of redefined clusters in each sample. **(D)** Two-dimensional scatter plots of KRT14 and KRT10 expression in all cell types. Note that there is no increasing fraction of KRT14$^{high}$ and KRT10$^{high}$ cells located in the right upper quadrant (RUQ) after long-term expansion. **(E)** Representative IF images of the nearby and expanded skin. KRT14 and KRT10 co-expressed cells (yellow with arrows) and the quantification of their fraction in epidermal cells.

## Long-Term Expansion Induced Decayed Proliferative Activities of the Epidermis

In contrast to the nby groups, the fraction of epidermal cells in exp groups identified by clusters showed comparable changes in numbers but displayed strikingly consistent reductions in the proportion of PC clusters (%) (F-Nby vs. F-Exp: 10.26 vs. 7.39 and N-Nby vs. N-Exp: 6.45 vs. 5.01) (**Figure 3A**), which was reminiscent of cell growth arrest after LTE (Wang et al., 2021). Furthermore, not all samples had a reduction in the number of basal stem cells after LTE, which was different from our expectations (**Figure 3A**). Proliferating epidermal cells (PC) were further subdivided into three subpopulations (PC1–3) based on the previously defined signature genes(Zou et al., 2021) (**Figures 3B,C**). Endowed with the largest number of cells, PC1 was defined by the high expression of suprabasal keratins KRT1 and KRT10, which are the earliest markers for keratinocytes' differentiation, indicating that renewal spinous cells were the most sensitive subpopulation to take on stretch-mediated regenerative state for long-term stretch. Also, the PC1 located in the suprabasal layer possessed the ability to respond to the acute inflammatory response reviewed by the GO enrichment analysis (**Figure 3D**). Combined with the highest expression of COL17A1 and KRT15, we inferred that PC2 was most likely the basal cells. We noticed that PC2 was enriched in upregulated GO in terms of negative regulation of cell proliferation and apoptosis. These upregulated GO terms were in agreement with the decaying tendency of cell proliferation in long-term expanded epidermis (**Figure 3D**). PC3 occupied the least proportion of proliferative cells with the highest expression of CALML3, PTN, and CYP27A1 (**Figures 3B,D**). According to the previous finding, it was regarded as the self-renewing state of the most quiescent basal cell and the inflammation-responsive subpopulation (Zou et al., 2021). Our enrichment confirmed that PC3 participated in epithelial differentiation, response to the cytokine, and energy production (aerobic respiration and the ATP metabolic process). According to previous findings, the daughter cells of proliferative cells discriminate in favor of renewal (KRT14 + KRT10-–) rather than commitment to differentiation (KRT14 + KRT10+) (Aragona et al., 2020). Therefore, further analysis of the PC cell group showed that it contains three subgroups that may represent proliferating cells biased for renewal (KRT14$^{hi}$KRT10$^{low}$), differentiation (KRT10$^{hi}$KRT14$^{low}$), and committed cells (KRT10$^{hi}$KRT14$^{hi}$) (**Figure 3E**). However, no consistent changes in the relative abundance of the renewal subgroups but a slight increase in the fraction of committed cells was observed in Exp vs. Nby comparisons for both sample

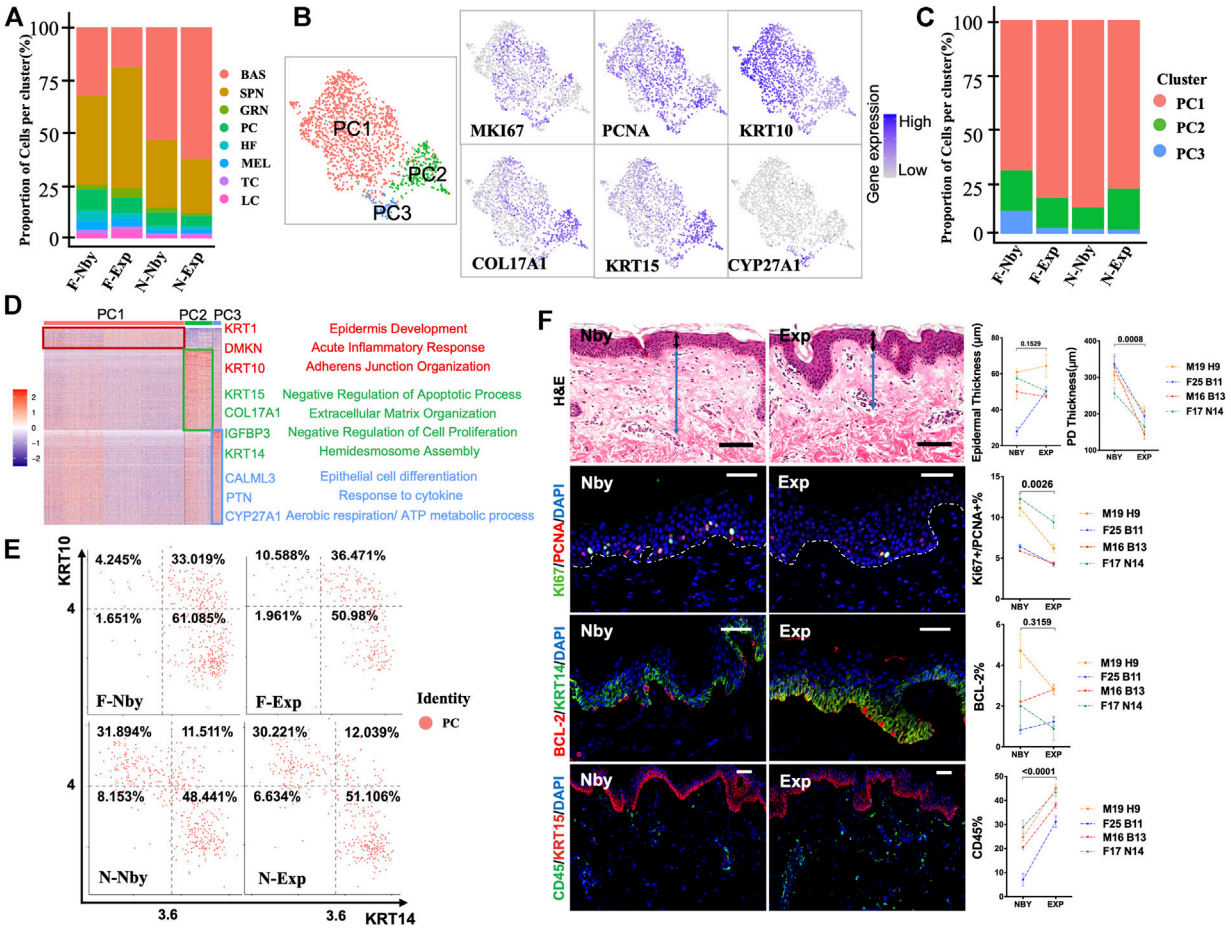


**FIGURE 3 |** LTE-induced decayed proliferative activities of the epidermis. **(A)** The proportion of cells for each of the eight cell types in each sample. Note the decreased fraction of the PC cell. **(B)** UMAP plots showing the subpopulations of proliferative cells (PC) with the expression of representative genes are shown to the right. **(C)** The proportion of the proliferative subpopulations in each sample. Note the inconsistent changes in the proportion between the samples. **(D)** Heatmap showing the scaled expression levels of genes highly expressed specifically in PC1, PC2, and PC3. The color key from blue to red indicates low to high gene expression levels. Cell-type-specific representative genes and GO terms are listed to the right. **(E)** Two-dimensional scatter plots of KRT14 and KRT10 expression in PC. **(F)** Representative images of the nby and exp skin. The sections belong to four individuals undergoing long-term tissue expansion. Two-way ANOVA was applied in statistical data analysis and $p < 0.05$ was considered statistically significant. Scale bars = 50 μM. All parameters were the same in all subsequent figures unless otherwise mentioned. First row: H&E-stained skin nearby, expansion skin, and quantification of the thickness of the epidermis, and papillary dermis (PD). A black bidirectional arrowhead refers to the thickness of the epidermis and a blue bidirectional arrowhead refers to the thickness of PD. Second row: IF staining images of proliferative KI67$^+$ and PCNA$^+$ cells and quantification of their percentage in epidermal cells. Third row: IF staining images of apoptotic BCL-2$^+$ cells and quantification of their percentage in epidermis cells. Last row: The inflammatory CD45$^+$ cells and the quantification of their fraction in dermal cells.

pairs after LTE, which is different from the RSE findings (**Figure 3E**). To verify these findings, we analyzed skin sections from additional independent LTE skin samples. H&E staining and immunofluorescence staining (IF) showed reduced thickness of the papillary dermis (PD) and increased CD45$^+$ immune cells in Exp vs. Nby skin (**Figure 3F**). These are consistent with the known properties of stretching skin (Zöllner et al., 2013; Aragona et al., 2020). However, the epidermal thickness after LTE was not constantly increased but reached a plateau or even decreased. To testify to the fraction of the PC subpopulation, staining of Ki67 and PCNA showed reduced expression of the proliferation marker in Exp vs. Nby skins (**Figure 3F**). Also, no comparable change in apoptosis (BCL-2$^+$) was detected, excluding the possibility of stretch-induced damage (**Figure 3F**). Overall, our finding provides an in-depth understanding of skin's adaption to long-term stretch-mediated regeneration and hints at the risk factors for complications of long-term expansion therapy.

## Identification of Transcriptomic Patterns Involved in the Regenerative Process of Long-Term Expansion

By Gene Set Enrichment Analysis (GSEA) analysis for significant enriched Msigdb hallmarks (Wu and Smyth, 2012) in the EDC group of each sample relative to other

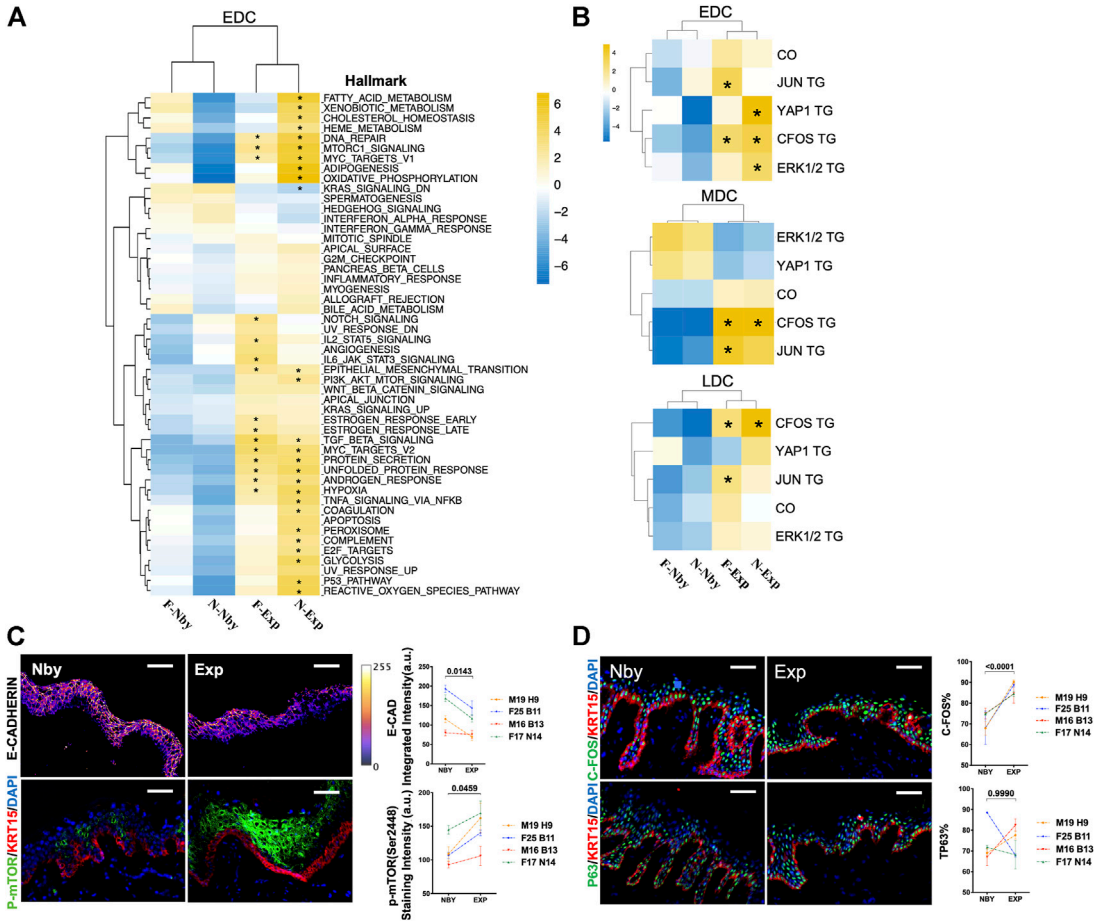


**FIGURE 4 |** Changes in the transcriptional profiles of the EDC subpopulation during LTE. **(A)** Heatmap showing the GSEA results of the MSigDB hallmark terms in EDC in four samples. $p < 0.05$ is considered statistically significant, same in all following figures unless otherwise mentioned. **(B)** Heatmap showing the GSEA results in of the previously published TGs of the transcriptional factors JUN, C-FOS, YAP1, and ERK1/2 cascade downstream signatures and GO terms of cytoskeleton organization. **(C)** Representative images of the E-CAD and p-mTOR staining in nby and exp samples from four other individuals and their quantification. The E-CAD IF staining intensity was shown by the pseudo-color integrated density signal. The color key (right) from purple to yellow indicates low to high integrated density signal levels. A.U, arbitrary units. **(D)** Representative IF images of the C-FOS and P63 staining in nby and exp skin and their quantification.

samples, we found that F-Exp and N-Exp EDCs are transcriptionally similar to each other but are distinct from F-Nby and N-Nby EDCs (**Figure 4A**), suggesting a specific transcriptional signature for epidermal stem cells under stretching. Among these, Exp EDC was significantly enriched for epithelial-mesenchymal transition (EMT), mTORC1 signaling, DNA repair, hypoxia, MYC targets, protein secretion and TGF-β signaling hallmarks, which partly are reported to be involved in epidermal stretching response (Liang et al., 2014; Zhou et al., 2020). Whereas the inflammatory infiltration during the long-term stretch was not enriched significantly, this was different from the highly inflammatory response of the short-term stretch (Aragona et al., 2020; Ledwon et al., 2020). The previous mouse study identified *AP-1, YAP, ERK* signaling, and cytoskeleton organization (CO) pathways as key drivers of stretching-induced skin growth *in vivo* (Aragona et al., 2020). To verify, we performed additional GSEA analysis using reported target genes (TG) of *C-FOS* (GSE10218, Durchdewald et al., 2008), *JUN* (GSE119762, Gago-Lopez et al., 2019), *YAP1* (GSE137531, Yuan et al., 2020), *ERK1/2* (GSE15417, Dumesic et al., 2009) and the GO ontology term of CO (7010, **Supplementary Table S1**). The results showed visible enrichment of most of these genesets in Exp vs. Nby EDCs (**Figure 4B**). Among these, C-FOS TGs are most consistently enriched in both Exp samples and also in MDC and LDC cell groups (**Figure 4B**). To verify these findings, we again performed IF analysis on independent clinical LTE samples. The data confirmed significant reduced E-CAD + expression (a classic EMT marker) (Zeisberg and Neilson, 2009), enhanced phospho-mTOR expression (activated mTORC1 signal marker) (Obara et al., 2015) (**Figure 4C**), and elevated C-FOS expression in Exp vs. Nby comparisons, while epidermal progenitor marker P63 (Soares and Zhou, 2018) remained unchanged (**Figure 4D**). Taken together, transcriptomic patterns are indeed altered jointly after LTE

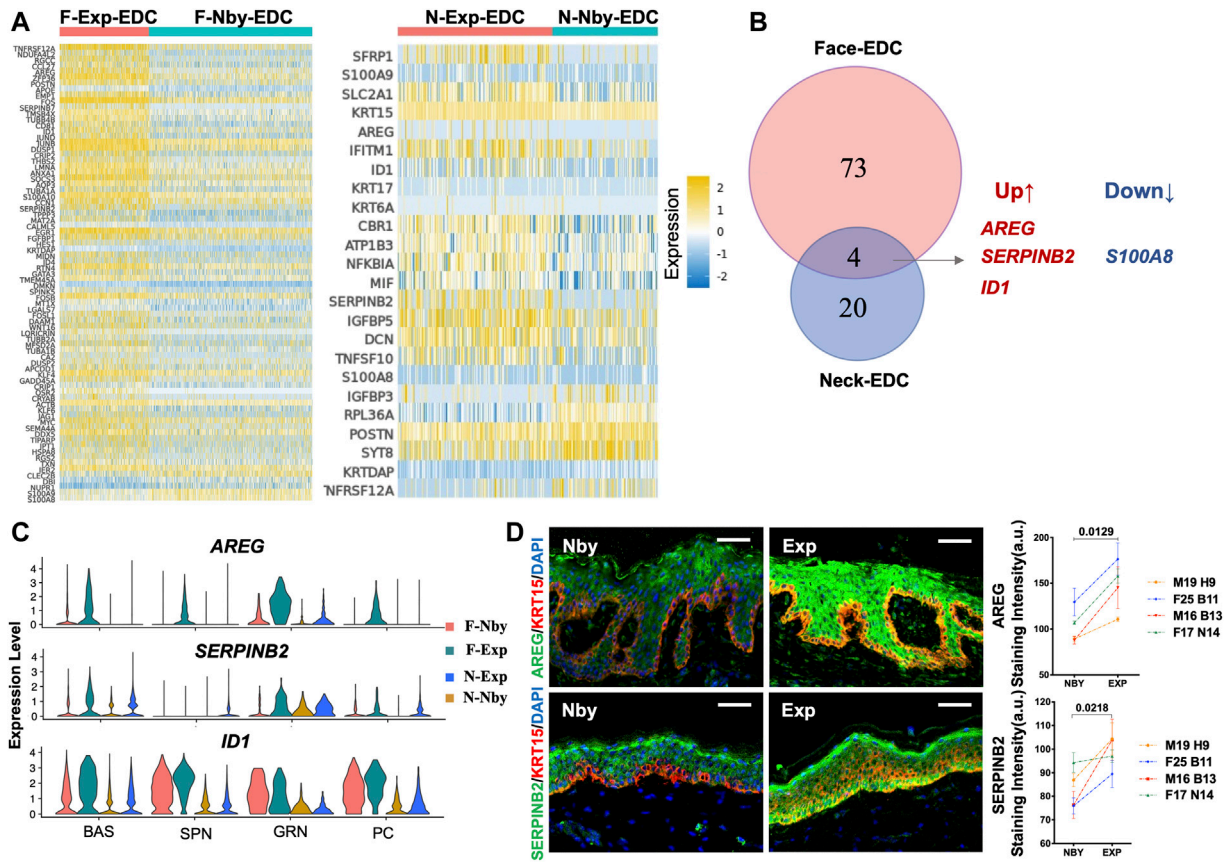


**FIGURE 5 |** Identification of the differentially expressed genes (DEGs) in the EDC subpopulation during LTE. **(A)** Heatmaps showing the DEGs identified in EDC between the exp and nby groups. The color key from blue to yellow indicates low to high gene expression levels. Representative genes are listed to the left (|FC| ≥ 0.5). **(B)** Venn diagram showing the overlap between DEGs in Face-EDC vs. Neck-EDC (|FC| ≥ 0.5). **(C)** Violin plots showing the expression level and distribution of the selected DEGs in subpopulations (BAS, SPN, GRN) and proliferative cells (PC) in four samples. **(D)** Representative IF images of the AREG and SERPINB2 staining in nearby and expanded skin from four other individuals and their quantification.

despite genetic specificities between the two samples used for scRNAseq.

## Gene Expression Changes in Human Epidermal EDC After Long-Term Expansion

To gain insights into the mechanisms of skin regeneration after LTE, we next examined the Exp vs. Nby changes of individual genes in the EDCs. Since the facial and cervical specimens were from different individuals and anatomic sites, we expected they would endow a high degree of heterogeneity between the samples. Differentially regulated genes (DEG) were considered as having an absolute fold change (|FC|) ≥ 0.5 (**Figure 5A**). As expected, the facial sample contained more DEGs than the cervical samples. Overlap between the DEGs, including only three upregulated genes and one downregulated gene, was observed in both sample pairs (**Figure 5B**). Accordingly, the violin plot shows the transcriptomic distribution of the upregulated genes (**Figure 5C**). IF analysis confirmed significant upregulation of Amphiregulin (AREG) and SERPINB2 in Exp vs. Nby comparison at the protein level (**Figure 5D**). Notably, *S100A8*, the downregulated gene, was the damage-associated molecular in skin disorders and will be secreted under inflammatory microenvironment (Defrêne et al., 2021). Its downregulation suggested the degree of inflammatory response was attenuated over time. Generally, whether they are novel regulators of clinical skin expansion would be an interesting topic for future analysis.

## Identification of Major Signaling Changes in Long-Term Expanded Human Epidermis

Signaling crosstalk via ligands and receptors is critical in tissue development and cellular decisions (Bhattacharjee et al., 2019). To clarify the underlying intercellular communications that drive skin regeneration for long-term expansion therapy, we analyzed intercellular communication networks among the 8 cell groups from scRNA-seq data using CellChat (Jin et al., 2021). It identified ligand-receptor pairs AREG-EGFR, CD96-NECTIN, and LAMIN-CD44 as the most upregulated significant signaling in exp versus nby counterparts, suggesting that these pathways are essential for stretch and likely contribute to the sustainability of long-term skin regeneration (**Figure 6A**). The downregulated

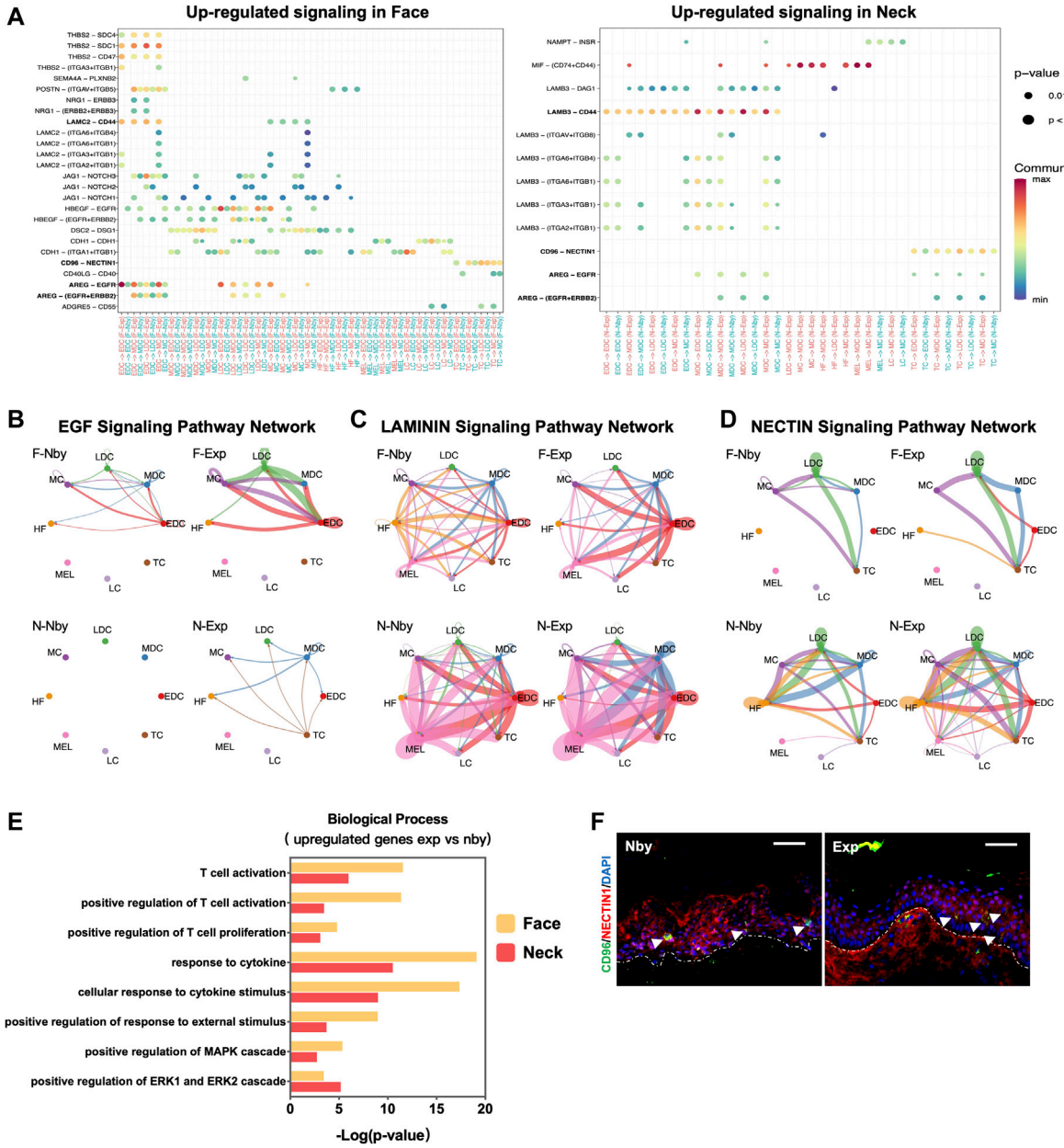


**FIGURE 6 |** Intercellular ligand–receptor prediction. **(A)** Visualization and comparison of the significant upregulated ligand-receptor pairs in the exp vs. nby groups. Dot color reflects communication probabilities, and dot size represents computed *p*-values. Empty space means the communication probability is zero. *p*-values are computed from a one-sided permutation test. **(B–D)** The inferred EGF, LAMIN, and NECTIN signaling networks. Different cell populations are color-coded. Circle size is proportional to the number of cells in each cell group, and edge width represents the communication probability. **(E)** GO biological process analysis of T cells. **(F)** Representative IF images of the NECTIN1 and CD96 staining in nby and exp skin. Arrows denote CD96 positive cells, and the dotted lines denote the basement membrane between the epidermis and dermis.

ligand-receptor pairs are shown in **Supplementary Figure S1A**. Cellchat revealed that the EGF pathway exhibited abundant signaling interactions. But EGF ligands in F-Exp are dominantly secreted by EDC, LDC, and MC, whereas in N-Exp they are secreted by MDC and TC (**Figure 6B**). LAMB3 and LAMC2 are the ligands of the laminin pathway that regulate the attachment of the basal membrane (Yap et al., 2019). The laminin-CD44 interaction contributes to the increased migration during wound healing (Michopoulou et al., 2020). Therefore, CellChat prediction suggests that LAMININN signaling in EDC and MDC cells plays a central role in directed migration after mechanical stretch (**Figure 6C**). CD96-NECTIN has been described as influencing the adhesive and migrative function of T cells and negatively controlling their cytokine responses (Seth et al., 2007; Chan et al., 2014, 96). Therefore, we predicted that epidermis-resident T cells may be

activated and interact with the epidermal cells during LTE (**Figure 6D**). To further elucidate the role of epidermis-resident T cells after LTE, we performed GO biological process enrichment analysis using the upregulated DEGs of T cells (log 2 (FC) > 0.3). The common terms of the two samples were listed in **Figure 6E**, including positive regulation of T cell activation and proliferation, response to inflammatory and immune responses, and positive regulation of MAPK and ERK cascade. The commonly upregulated genes include FOS and JUND, indicating the inflammatory regulation they may participate in LTE (**Supplementary Table S2**). We further performed IF staining to verify the bioinformatic analysis on cellular communication. As shown in **Figure 6F**, CD96-NECTIN1 ligand-receptor pairs from the bioinformatic analyses were verified experimentally. Overall, we predicted the biologically meaningful intercellular communications of human LTE from scRNA-seq data.

## DISCUSSION

Our study was designed as a paired study with control samples deriving from preferably comparable skin segments of the same subject. The sample size was adequate to yield a convincing conclusion, although the different anatomic sites exhibited distinct features. Our results not only highlighted the similarities across the spectrum of human LTE but also revealed consistently specific markers, including known signaling pathways and some new expansion-related genes. Functional studies are warranted to follow up on some of the unique molecular pathways identified in this work. Also, single-cell ATAC seq to unravel the chromatin landscape of basal stem cells requires further investigation in future analysis. It will also contribute to an enhanced understanding of the mechanisms of the LTE and the strategies to promote skin regeneration clinically.

Of note, Aragona et al. (2020) in Nature observed that increased stem-cell renewal fuels stem-cell differentiation and stretches the renewal-differentiation balance in favor of producing more stem cells. In our study, we investigated the renewal-differentiation balance of the proliferative cell based on the KRT14 and KRT10 expressions. Opposite to their findings, the renewal of proliferative cells did not show signs of increasing but the committed cells poised for differentiation did after LTE. Also, the thickening of the epidermis after the expansion is often accompanied by the activation of the differentiated process of the basal stem cell. However, we did not observe the increased fraction of the spinous cells or the enrichment of the $KRT14^{+}KRT10^{+}$ committed cells. We also expected the stem cell-like stretch cluster to be the fast-responding cluster once the stretch was applied. So, we performed Monocle3 and RNA velocity to predict whether a differentiation trajectory from basal stem cell to this "stretch" cluster exists in our LTE samples or not. However, our results elucidate that the skin composition remains in homeostasis after LTE. Overall, there are fundamental differences between RSE models and clinical MCVSE processes that need further investigation.

For more than 60 years (NEUMANN, 1957), physicians have taken advantage of the particular response and effective interventions of stretch-induced skin expansion. After years of stagnation, it is exciting to develop targets to promote regenerative competency and diminish the complications of LTE. Our finding showed that EMT was the key regulator to promoting regeneration and maintaining stemness in basal keratinocytes during LTE. It should be noted that the hypoxia (Thiery et al., 2009), DNA repair (Weyemi et al., 2016), TGF-β(Kahata et al., 2018), mTORC1 (Gulhati et al., 2011), MYC signal (Cho et al., 2010) were the upstream inducer that favors the activation of EMT process. All activities converge on the induction of the E-cadherin repressor, which has been verified at the protein level. Plus, the *AP-1* transcription factor comprises members of the *FOS* family (mainly *C-FOS*) and the *JUN* family (mainly *C-JUN* and *JUNB*) (Eferl and Wagner, 2003). *c-Fos* was reported to participate in the skin hyperplasia mediated by inflammation, suggesting that inflammation accompanied by LTE may trigger c-fos activation throughout this process (Briso et al., 2013). Additionally, the epidermal growth factor (EGF)-like molecular AREG plays a central role in orchestrating host protection (Zaiss et al., 2015) and epithelial regeneration (Lu et al., 2021). Accordingly, AREG has a huge potential to exert both anti-inflammatory and pro-regenerative effects and minimize the risk of complications during LTE. SerpinB2 maintains the barrier function of the stratum corneum (Schroder et al., 2016), suggesting its positive role in maintaining the integrity of the skin barrier.

It is noteworthy that tissue expansion results in the dermis thinned out (Agrawal and Agrawal, 2012), angiogenesis occurs (Zöllner et al., 2013), altered epithelial-mesenchymal interactions (Zhou et al., 2020, 1), chronic inflammation and fibroblast-myofibroblast differentiation (Kollmannsberger et al., 2018). The latter feature (differentiation of fibroblasts into myofibroblasts) is central to the excessive production of collagen-rich ECM (Tomasek et al., 2002; Tai et al., 2021) and dermal fibrosis (Gao et al., 2019; He et al., 2021) under mechanical stretch. A further study verified that the papillary dermis exerts crucial functions in the favorable prognosis of skin regeneration under stretch (Tan et al., 2022). It is also acknowledged that tissue-resident stem cells are critically important for the development and regeneration of the skin (Gonzales and Fuchs, 2017). Whereas stem cells derived from dermis including neural crest stem cells, MSC-like stem cells, and hematopoietic cells have a lower capacity to participate in the process of skin regeneration compared to epidermal stem cells and hair follicle stem cells (Díaz-García et al., 2021). What's more, the dermis interdigitates with the epidermis so that both the epithelium and mesenchyme release signal factors that regulate cellular behavior in a reciprocal manner (Russo et al., 2020). However, the presence of keratinocytes tips the balance of skin regeneration by directing ECM remodeling of fibroblasts (Ghaffari et al., 2009) and reconstructing the epithelial appendages (Wu et al., 2014). Thus, we assume that the epidermis plays a more vital role in skin regeneration compared to the dermis and have focused on the phenotypes of the epidermal stem cells in tissue expansion therapy.

However, the selection of the anatomical donor sites for skin expansion can vary considerably depending on different preoperative diagnoses and various operative procedures (Huang et al., 2011; Azzi et al., 2020). Because of the distinct transcriptional programming varied by the anatomical sites (Cheng et al., 2018), it is relatively challenging to exploit a common strategy to enhance the regenerative competency for long-term expansion therapy. Additionally, the expansion treatment process ends when the surface area of the expanded skin equals the sum of the surface area of the donor site plus the area of the adjacent soft-tissue defect, which means the endpoint of treatment meets the needs of surgical therapy rather than ends of the skin's expanded state. The study on the LTE samples under serial stretch did not reflect the early response of the expansion because any harvesting tissues during the expansion treatment process in the early or in the middle period were not able to meet ethical requirements and affect the patients' interests, including treatment safety and effects.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI; PRJNA797897.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human samples were from discarded plastic surgery specimens of healthy individuals with written informed consent. The procedures were in accordance with protocols approved by the Board of Ethical Review of Shanghai Ninth People's Hospital (2020-2018-129-T107-1) and the Ethics Committee of the Shanghai Institute of Nutrition and Health, and were in accordance with the Helsinki Declaration of 1975, as revised in 1983. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

CL, LZ, QL, and YS conceived and designed the research. CL performed cell isolation. YS performed the scRNA-seq data analysis. YS, LX, YL performed the IF staining analysis. YS wrote the original manuscript. KW prepared some parts of the tissue sections. LZ, CL, QL, YY and JL reviewed and edited the draft. Funding was acquired from QL, CL, LZ and YY. Human specimens were from JL, HL, YG, HZ, XH, YZ, and BW. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.865983/full#supplementary-material

**Supplementary Figure S1 | (A)** Visualization and comparison of the significant upregulated ligand-receptor pairs in the exp vs. nby groups. Dot color reflects communication probabilities, and dot size represents computed *p*-values. Empty space means the communication probability is zero. *p*-values are computed from a one-sided permutation test.

**Supplementary Table S1 |** List of genes used for GSEA analysis.

**Supplementary Table S2 |** List of jointly upregulated genes of T cells used for GO biological process analysis.

## REFERENCES

Agrawal, K., and Agrawal, S. (2012). Tissue Regeneration during Tissue Expansion and Choosing an Expander. *Indian J. Plast. Surg.* 45, 7–15. doi:10.4103/0970-0358.96566

Aragona, M., Sifrim, A., Malfait, M., Song, Y., Van Herck, J., Dekoninck, S., et al. (2020). Mechanisms of Stretch-Mediated Skin Expansion at Single-Cell Resolution. *Nature* 584, 268–273. doi:10.1038/s41586-020-2555-7

Asare, A., Levorse, J., and Fuchs, E. (2017). Coupling Organelle Inheritance with Mitosis to Balance Growth and Differentiation. *Science* 355, eaah4701. doi:10.1126/science.aah4701

Azzi, J. L., Thabet, C., Azzi, A. J., and Gilardino, M. S. (2020). Complications of Tissue Expansion in the Head and Neck. *Head Neck* 42, 747–762. doi:10.1002/hed.26017

Bergen, V., Lange, M., Peidli, S., Wolf, F. A., and Theis, F. J. (2020). Generalizing RNA Velocity to Transient Cell States through Dynamical Modeling. *Nat. Biotechnol.* 38, 1408–1414. doi:10.1038/s41587-020-0591-3

Bhattacharjee, O., Ayyangar, U., Kurbet, A. S., Ashok, D., and Raghavan, S. (2019). Unraveling the ECM-Immune Cell Crosstalk in Skin Diseases. *Front. Cel Dev. Biol.* 7, 68. doi:10.3389/fcell.2019.00068

Briso, E. M., Guinea-Viniegra, J., Bakiri, L., Rogon, Z., Petzelbauer, P., Eils, R., et al. (2013). Inflammation-mediated Skin Tumorigenesis Induced by Epidermal C-Fos. *Genes Dev.* 27, 1959–1973. doi:10.1101/gad.223339.113

Cao, J., Spielmann, M., Qiu, X., Huang, X., Ibrahim, D. M., Hill, A. J., et al. (2019). The Single-Cell Transcriptional Landscape of Mammalian Organogenesis. *Nature* 566, 496–502. doi:10.1038/s41586-019-0969-x

Chan, C. J., Martinet, L., Gilfillan, S., Souza-Fonseca-Guimaraes, F., Chow, M. T., Town, L., et al. (2014). The Receptors CD96 and CD226 Oppose Each Other in the Regulation of Natural Killer Cell Functions. *Nat. Immunol.* 15, 431–438. doi:10.1038/ni.2850

Cheng, J. B., Sedgewick, A. J., Finnegan, A. I., Harirchian, P., Lee, J., Kwon, S., et al. (2018). Transcriptional Programming of Normal and Inflamed Human Epidermis at Single-Cell Resolution. *Cel Rep.* 25, 871–883. doi:10.1016/j.celrep.2018.09.006

Cho, K. B., Cho, M. K., Lee, W. Y., and Kang, K. W. (2010). Overexpression of C-Myc Induces Epithelial Mesenchymal Transition in Mammary Epithelial Cells. *Cancer Lett.* 293, 230–239. doi:10.1016/j.canlet.2010.01.013

Defrêne, J., Berrazouane, S., Esparza, N., Pagé, N., Côté, M.-F., Gobeil, S., et al. (2021). Deletion of S100a8 and S100a9 Enhances Skin Hyperplasia and Promotes the Th17 Response in Imiquimod-Induced Psoriasis. *J.I.* 206, 505–514. doi:10.4049/jimmunol.2000087

Díaz-García, D., Filipová, A., Garza-Veloz, I., and Martinez-Fierro, M. L. (2021). A Beginner's Introduction to Skin Stem Cells and Wound Healing. *IJMS* 22, 11030. doi:10.3390/ijms222011030

Dumesic, P. A., Scholl, F. A., Barragan, D. I., and Khavari, P. A. (2009). Erk1/2 MAP Kinases Are Required for Epidermal G2/M Progression. *J. Cel Biol.* 185, 409–422. doi:10.1083/jcb.200804038

Durchdewald, M., Guinea-Viniegra, J., Haag, D., Riehl, A., Lichter, P., Hahn, M., et al. (2008). Podoplanin Is a Novel Fos Target Gene in Skin Carcinogenesis. *Cancer Res.* 68, 6877–6883. doi:10.1158/0008-5472.CAN-08-0299

Eferl, R., and Wagner, E. F. (2003). AP-1: a Double-Edged Sword in Tumorigenesis. *Nat. Rev. Cancer* 3, 859–868. doi:10.1038/nrc1209

Ellis, S. J., Gomez, N. C., Levorse, J., Mertz, A. F., Ge, Y., and Fuchs, E. (2019). Distinct Modes of Cell Competition Shape Mammalian Tissue Morphogenesis. *Nature* 569, 497–502. doi:10.1038/s41586-019-1199-y

Fuchs, E. (2008). Skin Stem Cells: Rising to the Surface. *J. Cel. Biol.* 180, 273–284. doi:10.1083/jcb.200708185

Gago-Lopez, N., Mellor, L. F., Megías, D., Martín-Serrano, G., Izeta, A., Jimenez, F., et al. (2019). Role of Bulge Epidermal Stem Cells and TSLP Signaling in Psoriasis. *EMBO Mol. Med.* 11, e10697. doi:10.15252/emmm.201910697

Gao, Y., Zhou, J., Xie, Z., Wang, J., Ho, C.-k., Zhang, Y., et al. (2019). Mechanical Strain Promotes Skin Fibrosis through LRG-1 Induction Mediated by ELK1 and ERK Signalling. *Commun. Biol.* 2, 359. doi:10.1038/s42003-019-0600-6

Ghaffari, A., Kilani, R. T., and Ghahary, A. (2009). Keratinocyte-Conditioned Media Regulate Collagen Expression in Dermal Fibroblasts. *J. Invest. Dermatol.* 129, 340–347. doi:10.1038/jid.2008.253

Gonzales, K. A. U., and Fuchs, E. (2017). Skin and its Regenerative Powers: An Alliance between Stem Cells and Their Niche. *Dev. Cel.* 43, 387–401. doi:10.1016/j.devcel.2017.10.001

Gulhati, P., Bowen, K. A., Liu, J., Stevens, P. D., Rychahou, P. G., Chen, M., et al. (2011). mTORC1 and mTORC2 Regulate EMT, Motility, and Metastasis of Colorectal Cancer via RhoA and Rac1 Signaling Pathways. *Cancer Res.* 71, 3246–3256. doi:10.1158/0008-5472.CAN-10-4058

He, J., Fang, B., Shan, S., Xie, Y., Wang, C., Zhang, Y., et al. (2021). Mechanical Stretch Promotes Hypertrophic Scar Formation through Mechanically Activated Cation Channel Piezo1. *Cell Death Dis.* 12, 226. doi:10.1038/s41419-021-03481-6

Huang, X., Qu, X., and Li, Q. (2011). Risk Factors for Complications of Tissue Expansion: A 20- Year Systematic Review and Meta-Analysis. *Plast. Reconstr. Surg.* 128, 787–797. doi:10.1097/PRS.0b013e3182221372

Huang, X., Liang, X., Zhou, Y., Li, H., Du, H., Suo, Y., et al. (2021). CDH1 Is Identified as A Therapeutic Target for Skin Regeneration after Mechanical Loading. *Int. J. Biol. Sci.* 17, 353–367. doi:10.7150/ijbs.51309

Jin, S., Guerrero-Juarez, C. F., Zhang, L., Chang, I., Ramos, R., Kuan, C.-H., et al. (2021). Inference and Analysis of Cell-Cell Communication Using CellChat R Package. *Nat. Commun.* 12, 1088. doi:10.1038/s41467-021-21246-9

Kahata, K., Dadras, M. S., and Moustakas, A. (2018). TGF-β Family Signaling in Epithelial Differentiation and Epithelial-Mesenchymal Transition. *Cold Spring Harb Perspect. Biol.* 10, a022194. doi:10.1101/cshperspect.a022194

Kollmannsberger, P., Bidan, C. M., Dunlop, J. W. C., Fratzl, P., and Vogel, V. (2018). Tensile Forces Drive a Reversible Fibroblast-To-Myofibroblast Transition during Tissue Growth in Engineered Clefts. *Sci. Adv.* 4, eaao4881. doi:10.1126/sciadv.aao4881

Le, H. Q., Ghatak, S., Yeung, C.-Y. C., Tellkamp, F., Günschmann, C., Dieterich, C., et al. (2016). Mechanical Regulation of Transcription Controls Polycomb-Mediated Gene Silencing during Lineage Commitment. *Nat. Cel. Biol.* 18, 864–875. doi:10.1038/ncb3387

Ledwon, J. K., Kelsey, L. J., Vaca, E. E., and Gosain, A. K. (2020). Transcriptomic Analysis Reveals Dynamic Molecular Changes in Skin Induced by Mechanical Forces Secondary to Tissue Expansion. *Sci. Rep.* 10, 15991. doi:10.1038/s41598-020-71823-z

Lee, T., Vaca, E. E., Ledwon, J. K., Bae, H., Topczewska, J. M., Turin, S. Y., et al. (2018). Improving Tissue Expansion Protocols through Computational Modeling. *J. Mech. Behav. Biomed. Mater.* 82, 224–234. doi:10.1016/j.jmbbm.2018.03.034

Liang, Y. M., Huang, X. L., Chen, G., Sheng, L. L., and Li, Q. F. (2014). Activated Hypoxia-inducible Factor-1α Pathway Modulates Early Events in Stretch-induced Skin Neovascularization via Stromal Cell-derived Factor-1 and Vascular Endothelial Growth Factor. *Br. J. Dermatol.* 171, 996–1005. doi:10.1111/bjd.12920

Lohana, P., Moiemen, N. S., and Wilson, Y. T. (2012). The Use of Osmed(TM) Tissue Expanders in Paediatric burns Reconstruction. *Ann. Burns Fire Disasters* 25, 38–42.

Lu, Q., Gao, Y., Fan, Z., Xiao, X., Chen, Y., Si, Y., et al. (2021). Amphiregulin Promotes Hair Regeneration of Skin-derived Precursors via the PI3K and MAPK Pathways. *Cell Prolif.* 54, e13106. doi:10.1111/cpr.13106

Michopoulou, A., Montmasson, M., Garnier, C., Lambert, E., Dayan, G., and Rousselle, P. (2020). A Novel Mechanism in Wound Healing: Laminin 332 Drives MMP9/14 Activity by Recruiting Syndecan-1 and CD44. *Matrix Biol.* 94, 1–17. doi:10.1016/j.matbio.2020.06.004

Nava, M. M., Miroshnikova, Y. A., Biggs, L. C., Whitefield, D. B., Metge, F., Boucas, J., et al. (2020). Heterochromatin-Driven Nuclear Softening Protects the Genome against Mechanical Stress-Induced Damage. *Cell* 181, 800–817.e22. doi:10.1016/j.cell.2020.03.052

Neumann, C. G. (1957). The Expansion of an Area of Skin by Progressive Distention of a Subcutaneous Balloon. *Plast. Reconstr. Surg.* 19, 124–130. doi:10.1097/00006534-195702000-00004

Obara, I., Medrano, M. C., Signoret-Genest, J., Jiménez-Díaz, L., Géranton, S. M., and Hunt, S. P. (2015). Inhibition of the Mammalian Target of Rapamycin Complex 1 Signaling Pathway Reduces Itch Behaviour in Mice. *Pain* 156, 1519–1529. doi:10.1097/j.pain.0000000000000197

Obdeijn, M. C., Nicolai, J.-P. A., and Werker, P. M. N. (2009). The Osmotic Tissue Expander: a Three-Year Clinical Experience. *J. Plast. Reconstr. Aesthet. Surg.* 62, 1219–1222. doi:10.1016/j.bjps.2007.12.088

Russo, B., Brembilla, N. C., and Chizzolini, C. (2020). Interplay between Keratinocytes and Fibroblasts: A Systematic Review Providing a New Angle for Understanding Skin Fibrotic Disorders. *Front. Immunol.* 11, 648. doi:10.3389/fimmu.2020.00648

Schroder, W. A., Anraku, I., Le, T. T., Hirata, T. D. C., Nakaya, H. I., Major, L., et al. (2016). SerpinB2 Deficiency Results in a Stratum Corneum Defect and Increased Sensitivity to Topically Applied Inflammatory Agents. *Am. J. Pathol.* 186, 1511–1523. doi:10.1016/j.ajpath.2016.02.017

Seth, S., Maier, M. K., Qiu, Q., Ravens, I., Kremmer, E., Förster, R., et al. (2007). The Murine pan T Cell Marker CD96 Is an Adhesion Receptor for CD155 and

Nectin-1. *Biochem. Biophys. Res. Commun.* 364, 959–965. doi:10.1016/j.bbrc.2007.10.102

Soares, E., and Zhou, H. (2018). Master Regulatory Role of P63 in Epidermal Development and Disease. *Cell. Mol. Life Sci.* 75, 1179–1190. doi:10.1007/s00018-017-2701-z

Tai, Y., Woods, E. L., Dally, J., Kong, D., Steadman, R., Moseley, R., et al. (2021). Myofibroblasts: Function, Formation, and Scope of Molecular Therapies for Skin Fibrosis. *Biomolecules* 11, 1095. doi:10.3390/biom11081095

Tan, P.-C., Zhou, S.-B., Ou, M.-Y., He, J.-Z., Zhang, P.-Q., Zhang, X.-J., et al. (2022). Mechanical Stretching Can Modify the Papillary Dermis Pattern and Papillary Fibroblast Characteristics during Skin Regeneration. *J. Invest. Dermatol.* S0022202X22001178 Online ahead of print. doi:10.1016/j.jid.2021.11.043

Thiery, J. P., Acloque, H., Huang, R. Y. J., and Nieto, M. A. (2009). Epithelial-Mesenchymal Transitions in Development and Disease. *Cell* 139, 871–890. doi:10.1016/j.cell.2009.11.007

Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., and Brown, R. A. (2002). Myofibroblasts and Mechano-Regulation of Connective Tissue Remodelling. *Nat. Rev. Mol. Cel Biol.* 3, 349–363. doi:10.1038/nrm809

Topczewska, J. M., Ledwon, J. K., Vaca, E. E., and Gosain, A. K. (2019). Mechanical Stretching Stimulates Growth of the Basal Layer and Rete Ridges in the Epidermis. *J. Tissue Eng. Regen. Med.* 13, 2121–2125. doi:10.1002/term.2952

Wang, J., Zhang, Y., Gao, Y., Shan, S., and Li, Q. (2021). EZH2 Regulates the Correlation between Skin Regeneration and the Duration of Mechanical Stretch. *J. Invest. Dermatol.* 141, 894–902.e9. doi:10.1016/j.jid.2020.09.007

Weyemi, U., Redon, C. E., and Bonner, W. M. (2016). H2AX and EMT: Deciphering beyond DNA Repair. *Cell Cycle* 15, 1305–1306. doi:10.1080/15384101.2016.1160659

Wu, D., and Smyth, G. K. (2012). Camera: a Competitive Gene Set Test Accounting for Inter-gene Correlation. *Nucleic Acids Res.* 40, e133. doi:10.1093/nar/gks461

Wu, X., Scott, L., Washenik, K., and Stenn, K. (2014). Full-Thickness Skin with Mature Hair Follicles Generated from Tissue Culture Expanded Human Cells. *Tissue Eng. A* 20, 3314–3321. doi:10.1089/ten.tea.2013.0759

Yap, L., Tay, H. G., Nguyen, M. T. X., Tjin, M. S., and Tryggvason, K. (2019). Laminins in Cellular Differentiation. *Trends Cel Biol.* 29, 987–1000. doi:10.1016/j.tcb.2019.10.001

Yuan, Y., Park, J., Feng, A., Awasthi, P., Wang, Z., Chen, Q., et al. (2020). YAP1/TAZ-TEAD Transcriptional Networks Maintain Skin Homeostasis by Regulating Cell Proliferation and Limiting KLF4 Activity. *Nat. Commun.* 11, 1472. doi:10.1038/s41467-020-15301-0

Zaiss, D. M. W., Gause, W. C., Osborne, L. C., and Artis, D. (2015). Emerging Functions of Amphiregulin in Orchestrating Immunity, Inflammation, and Tissue Repair. *Immunity* 42, 216–226. doi:10.1016/j.immuni.2015.01.020

Zeisberg, M., and Neilson, E. G. (2009). Biomarkers for Epithelial-Mesenchymal Transitions. *J. Clin. Invest.* 119, 1429–1437. doi:10.1172/JCI36183

Zhou, Y., Li, H., Liang, X., Du, H., Suo, Y., Chen, H., et al. (2020). The CCN1 (CYR61) Protein Promotes Skin Growth by Enhancing Epithelial-mesenchymal Transition during Skin Expansion. *J. Cel. Mol. Med.* 24, 1460–1473. doi:10.1111/jcmm.14828

Zhu, H., Gao, Z., Tremp, M., Zan, T., Li, Q., Xie, F., et al. (2018). Application of Tissue Expansion with Perforator Flaps for Reconstruction of Challenging Skin Lesions. *J. Reconstr. Microsurg.* 34, 013–020. doi:10.1055/s-0037-1606122

Zöllner, A. M., Holland, M. A., Honda, K. S., Gosain, A. K., and Kuhl, E. (2013). Growth on Demand: Reviewing the Mechanobiology of Stretched Skin. *J. Mech. Behav. Biomed. Mater.* 28, 495–509. doi:10.1016/j.jmbbm.2013.03.018

Zou, Z., Long, X., Zhao, Q., Zheng, Y., Song, M., Ma, S., et al. (2021). A Single-Cell Transcriptomic Atlas of Human Skin Aging. *Dev. Cel.* 56, 383–397.e8. doi:10.1016/j.devcel.2020.11.002

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