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REVIEW

Application of dental stem cells in three-dimensional tissue regeneration

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Abstract

Dental stem cells can differentiate into different types of cells. Dental pulp stem cells, stem cells from human exfoliated deciduous teeth, periodontal ligament stem cells, stem cells from apical papilla, and dental follicle progenitor cells are five different types of dental stem cells that have been identified during different stages of tooth development. The availability of dental stem cells from discarded or removed teeth makes them promising candidates for tissue engineering. In recent years, three-dimensional (3D) tissue scaffolds have been used to reconstruct and restore different anatomical defects. With rapid advances in 3D tissue engineering, dental stem cells have been used in the regeneration of 3D engineered tissue. This review presents an overview of different types of dental stem cells used in 3D tissue regeneration, which are currently the most common type of stem cells used to treat human tissue conditions.

Key Words: Dental stem cells; Dental pulp stem cells; Stem cells from human exfoliated deciduous teeth; Periodontal ligament stem cells; Stem cells from apical papilla; Dental

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Core Tip: Dental stem cell seeding in three-dimensional (3D) engineered scaffolds that mimic the human tissue microenvironment is an emerging technology for regenerative medicine. Dental pulp stem cells, stem cells from human exfoliated deciduous teeth, periodontal ligament stem cells, stem cells from apical papilla, and dental follicle progenitor cells have been used for tissue regeneration utilizing 3D approaches. The analytical results of this literature review reveal many basic and preclinical studies that support the hypothesis that the application of dental stem cells is a feasible approach for translational medicine and is an applicable method for 3D tissue regeneration.

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INTRODUCTION

The multipotent properties of stem cells make them excellent sources of material for tissue repair. Five dental-derived cell types have been isolated and characterized as dental stem cells[1]. Dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP), and dental follicle progenitor cells (DFPCs) are different types of dental stem cells involved in different stages of tooth development (Figure 1). Considering their differentiation potential, dental stem cells have been introduced to regenerate damaged or lost tissue. Dental stem cells are not restricted to use in dental tissue repair but can also participate in neural, adipose, bone, and cartilage tissue regeneration[2,3]. Recently, three-dimensional (3D) tissue engineering has been applied to therapeutic medicine. Cells are seeded in 3D engineered scaffolds to mimic the human tissue microenvironment during cell differentiation. The cell morphology and gene expression of the cells cultured under 3D conditions are more consistent with those of cells observed in native tissue[4]. The use of customized 3D tooth implants with dental stem cells seeded in suitable scaffolds as replacements for lost teeth is a promising approach in dentistry. In addition to tooth repair, there is growing interest in the concept of 3D tissue regeneration with dental stem cells.

Here, we searched databases to identify the literature on dental stem cells used in 3D tissue regeneration. The literature searches and data mining were performed by customized scripts with the "easyPubMed" and "PubMed.mineR" packages in R for use with the PubMed database[5-7]. The keywords used in the queries included "pulp stem cells", "exfoliated deciduous teeth stem cell", "periodontal ligament stem cell", "apical papilla", "dental follicle cells", "3D", "tissue", "regeneration" and "engineering". The search results were output with the "abstract" format in the "easyPubMed" package and were analyzed by the "PubMed.minR" package. A total of 88 papers were found with the aforementioned criteria. After review, only one-third of the papers articulated original research on dental stem cells in 3D tissue regeneration. In this review, we aim to provide a clear point of view on each type of dental stem cell used in combination with 3D tissue scaffolds, such as microspheres, hydrogels, or 3D printed scaffolds, to regenerate into teeth, neurons, bone, blood vessels and cartilage (Figure 1 and Table 1).

DPSCS

DPSCs located in the soft connective tissue inside the dental crown were first identified in 2000 (Figure 1)[8]. DPSCs exhibit MSC-like properties, including a high proliferation rate, multilineage potential, and immunomodulatory properties[8,9]. Even though DPSCs exhibit features similar to those of BMSCs, their characteristics of

Table 1 List of dental stem cells used for three-dimensional tissue regeneration

Dental stem cells	Biomaterials	Addition of materials/growth factors/cells	Type of tissue regeneration	Ref.
DPSCs				
DPSCs	CaP porous granules, NF-gelatin/MgP	No	Odontogenic differentiation	Nam *et al*[24], and Qu *et al*[30]
	SS-PLLA-b-PLYS, pNIPAAm, NF-PLLA	No	Pulp-dentin regeneration	Kuang *et al*[16], Itoh *et al*[17], and Soares *et al*[18]
	Coll/HA/PLCL, ABM/ABM-P-15, PVA/PU	No	Bone tissue	Mohanram *et al*[13], Cooke *et al*[19], and Akkouch *et al*[21]
	OMMT/PVA	No	Neuro-like cells	Ghasemi Hamidabadi *et al*[47]
	Matrigel	No	Endotheliocytes and pericytes	Luzuriaga *et al*[51]
	Collagen gel	SDF1, bFGF	Dental pulp tissue	Suzuki *et al*[23]
		BMP7		
DPSCs with growth factors	Ti6Al4V	Poly-L-lys coating	Osteoblastic differentiation	Galli *et al*[32]
	Porous silk fibroin	bFGF	Dental pulp tissue	Yang *et al*[26]
	PCL	VEGF, BMP2	Vascularized bone tissue	Park *et al*[39]
	HP hydrogel	bFGF	Spinal cord	Luo *et al*[48]
DPSCs with other cells	Matrigel and collagen gel	Human normal oral epithelial cells	Epithelium invagination-like structure	Xiao and Tsutsui[35]
	PCL/PLDLA	Endothelial cells	Vascularized bone tissue	Jin and Kim[36]
	PLLA/PLGA	Human neonatal dermal fibroblasts	Spinal cord	Guo *et al*[50]
DPSCs in 3D printed scaffolds	HA/TCP	Apical papilla (SCAP)	Pulp-dentin regeneration	Hilkens *et al*[40]
	PCL	Platelet-rich plasma	Calvaria bone	Li *et al*[27]
	Alg-Gel		Bone	Yu *et al*[38]
	PLAS		Neural differentiation	Hsiao *et al*[42]
	AMP/ECM		Craniomaxillofacial bone	Dubey *et al*[41]
SHED				
SHED with growth factors	No	EGF, FGF	Spinal cord	Feng *et al*[58]
	No	SHED-conditioned medium	Sciatic nerve	Sugimura-Wakayama *et al*[59]
SHED in 3D formed scaffolds	Polylactoglycolide, SHED aggregated hemisphere		Bone tissue	Laino *et al*[56], and Vakhrushev *et al*[57]
PDLSCs				
PDLSCs	Hydroxyapatite/β-tricalcium phosphate (HA/β-TCP)		Periodontal tissue	Kim *et al*[66]
	GelMA/PEG		PDLSC proliferation	Ma *et al*[80]
PDLSCs with growth factors	PLGA	CTGF, BMP-7, BMP-2	Periodontal tissue	Cho *et al*[73]
	Platelet-rich fibrin	Aspirin	Periodontal tissue	Du *et al*[76]
PDLSCs with other cells	Collagen/Chitosan	Somatic MSCs and DPSCs	Odontogenic differentiation	Ravindran *et al*[79]
	No	HUVECs	Periodontal tissue	Kramer[77]
	PLGA–PEG–PLGA thermal hydrogel	PDLSCs overexpressing PDGF-BB	Alveolar bone tissue	Pan *et al*[78]

SCAP				
SCAP with growth factors	PLLA nanofibrous microspheres (NF-MS)	BMP-2	Pulp-dentin regeneration	Wang *et al*[86]
	No	BMP-2, SDF-1α	Odontoblast differentiation	Xiao *et al*[87]
	Alg-Dent hydrogel	Dentin ECM	Pulp-dentin regeneration	Athirasala *et al*[95]
DFPCs				
DFPCs	Coll-nano-HA/OPS		Bone tissue	Salgado *et al*[102]

DFPCs: Dental follicle progenitor cells; SCAP: Stem cells from apical papilla; PDLSCs: Periodontal ligament stem cells; SHED: Human exfoliated deciduous teeth; DPSCs: Dental pulp stem cells; SDF1: Stromal-derived factor-1α; bFGF: Basic fibroblast growth factor; BMP-7: Bone morphogenetic protein-7; Ti6Al4V: Titanium-6-aluminum-4-vanadium; Poly-L-lys: Poly-L-lysine; CaP: Calcium phosphate; OECs: Human normal oral epithelial cells; PLCL: Collagen (Coll)/hydroxyapatite (HA)/poly(l-lactide-coε-caprolactone); NF-gelatin/MgP: Gelatin/magnesium phosphate; VEGF: Vascular endothelial growth factor; BMP-2: Morphogenetic protein-2; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; PCL: Polycaprolactone; NF-SMS: Nanofibrous spongy microspheres; SS-PLLA-b-PLYS: Star-shaped poly(l-lactic acid)-block-poly(l-lysine); PLDLA: Poly-L/D-lactide; ECs: Endothelial cells; HA/TCP: Hydroxyapatite/tricalcium phosphate; OMMT/PVA: Chitosan-intercalated montmorillonite/poly(vinyl alcohol); PRP: Platelet-rich plasma; pNIPAAm: Poly-N-isopropylacrylamide gel; HP: Heparin-poloxamer hydrogel; NF-PLLA: Nanofibrous poly(l-lactic acid) scaffolds; Alg-Gel: Alginate/gelatin hydrogel; 3DP-PLASs: Polylactic acid scaffolds; ABM: Bone mineral; ABM-P-15: Biomimetic collagen peptide; PVA: Polyvinyl alcohol; PU: Polyurethane; AMPs: Amorphous magnesium phosphates; ECM: Extracellular matrix; PLLA: Polylactoglycolide scaffolds; NF-MS: Nanofibrous microspheres; SDF-1α: Normal cell-derived factor-1α; GelMA: Gelatin methacrylate; PEG: Poly(ethylene glycol); dimethacrylate; PLGA: Poly(lactic-co-glycolic acids); CTGF: Connective tissue growth factor; HUVECs: Human umbilical vein endothelial cells; Coll-nano-HA/OPS: Collagen-nanohydroxyapatite/phosphoserine.

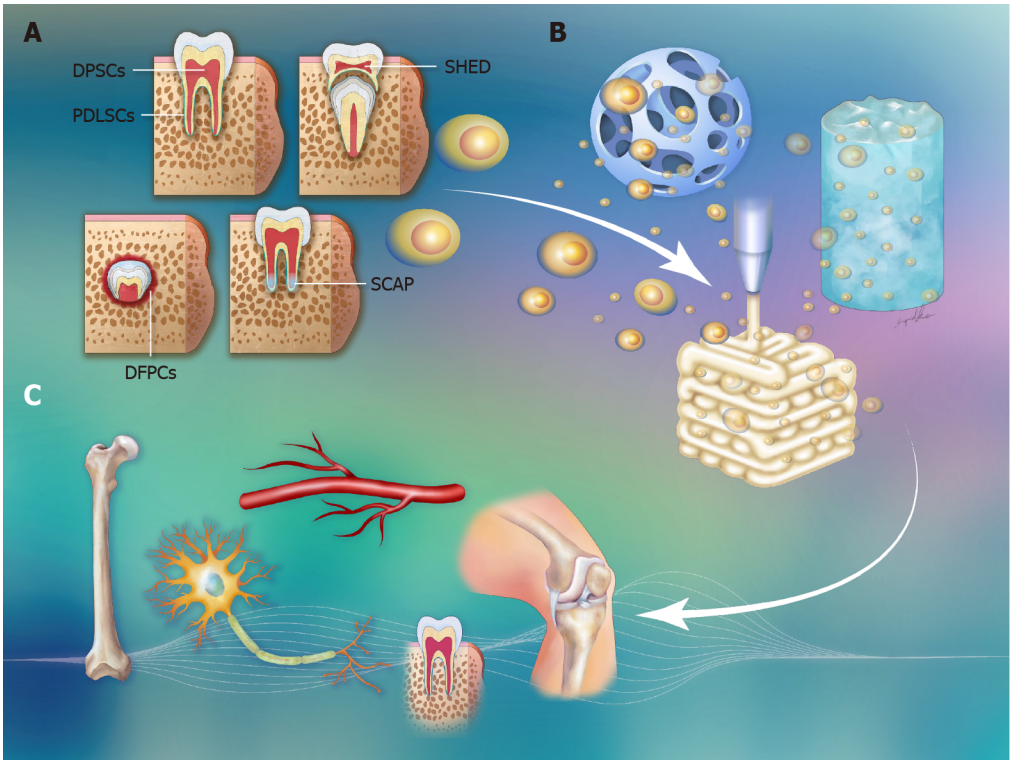

Figure 1 Schematic illustration of dental stem cells in three-dimensional tissue regeneration. A: Five different types of dental stem cells are harvested during different tooth developmental stages; B: Dental stem cells are incorporated with various forms of three-dimensional (3D) biomaterials (microspheres, hydrogels, or 3D printed scaffolds) to generate 3D engineered tissue; C: Dental stem cells are induced to differentiate into different types of tissue, such as teeth, neurons, bone, blood vessels and cartilage.

causing little morbidity at the donor site, a higher proliferation rate, and multipotency make DPSCs better stem cell sources for tissue regeneration[10]. DPSCs cocultured with apical bud cells (ABCs) exhibited more active odontogenic differentiation ability than BMSCs cocultured with ABCs[11]. The neural differentiation of IMR-32 cells was significantly enhanced when treated with secretomes derived from DPSCs compared to BMSCs[12]. The assessment of neurogenic potential on the secretome of DPSCs and BMSCs indicated that DPSCs presented better potential for neural differentiation[12]. Most DPSC studies have focused on dental pulp and bone tissue regeneration.

Compared to bone marrow stem cells, DPSCs have a higher proliferation rate and better osteogenic capacity when seeded in a scaffold of bone mineral (ABM) coated with a biomimetic collagen peptide (ABM-P-15), even generating a more organized collagenous matrix 8 wk after *in vivo* implantation[13]. Moreover, different gene expression patterns have been found in the transcriptome profiles of DPSCs compared to those of bone marrow stem cells, indicating unique gene expression patterns within DPSCs[14].

Application in 3D tissue regeneration

In addition to conventional tissue regeneration approaches with cells loaded on two-dimensional scaffolds, DPSCs have been cultured on 3D biomaterials for the development of tissue constructs. A bioink containing human DPSCs and fibrinogen incorporated with polycaprolactone (PCL) was designed for the production of dentin pulp complex structures[15]. Nanofibrous spongy microspheres made from star-shaped poly(l-lactic acid)-block-poly(l-lysine) (SS-PLLA-b-PLYS) were seeded with DPSCs for dental pulp tissue regeneration[16]. Poly-N-isopropyl acrylamide (pNIPAAm) gel containing DPSCs was made in a rod shape to fill in the root canal for pulp tissue regeneration[17]. Simvastatin and nanofibrous poly(l-lactic acid) (NF-PLLA) scaffolds[18] and a mixture of polyvinyl alcohol (PVA) and polyurethane (PU) [19] were combined with DPSCs to investigate the potential of tissue regeneration. Self-assembling peptides, with structures similar to the extracellular matrix (ECM), are among the smart materials used for 3D culture[20]. A 3D scaffold composed of collagen (Coll), hydroxyapatite (HA), and poly(L-lactide-co"-caprolactone) (PLCL) increased the adhesion and viability of DPSCs and enhanced bone regeneration compared to a PLCL-only scaffold[21]. DPSCs grown in a peptide-based scaffold presented RGD- and vascular endothelial growth factor (VEGF)-mimetic peptide epitopes and exhibited better survival and angiogenic and odontogenic differentiation [22].

With increased knowledge of the function of growth factors, an increasing number of studies have introduced growth factors into different types of tissue regeneration. In 2011, human DPSCs were placed on the surface of 3D collagen cylinders and cultured with the addition of stromal-derived factor-1α, basic fibroblast growth factor (bFGF), and bone morphogenetic protein-7 (BMP-7) for dental pulp regeneration[23]. Seeding DPSCs on 3D calcium phosphate (CaP) porous granules promoted odontogenic differentiation by increasing the gene expression of dentin sialophosphoprotein (*DSPP*) and dentin matrix protein 1 (*DMP1*)[24]. Porous silk fibroin scaffolds fabricated with bFGF, which has been reported to facilitate pulp regeneration[25], were used to fill the root canal space for tooth repair[26]. Platelet-rich plasma (PRP) containing various growth factors along with DPSCs was added to 3D printed PCL mesh for bone regeneration in a rat calvaria defect model[27].

In addition to growth factors, metal ions have also been confirmed to contribute to cell differentiation[28]. Magnesium (Mg) is involved in the process of biomineralization during bone and tooth development[29]. Qu *et al*[30] incorporated Mg into nanofibrous gelatin biomaterials to develop 3D gelatin/Mg phosphate (NF-gelatin/MgP) scaffolds seeded with DPSCs, and odontogenic proliferation and differentiation were enhanced. The materials used for dental implants, such as titanium-6-aluminum-4-vanadium (Ti6Al4V), are also used as 3D scaffolds for tissue regeneration. Their properties of low corrosion and smooth metal surfaces prevent stem cells from colonizing this biomaterial[31]. Coatings of poly-L-lysine (poly-L-lys), which carries positive charges, induced focal adhesion kinase activation and increased the osteoblastic differentiation of hDPSCs[32]. A coculture system not only provides intercellular factors but also enables communication between two types of cells, which is critical for the development and arrangement of the ECM[33,34]. DPSCs cocultured with human normal oral epithelial cells harvested from gingival tissue were inoculated into 3D Matrigel to form an epithelium invagination-like structure, a key feature of early tooth development[35]. Poly-L/D-lactide (PCL/PLDLA) porous microspheres were loaded with DPSCs and human endothelial cells to promote osteogenesis and angiogenesis for vascularized bone tissue regeneration[36].

3D printing techniques can print cells, growth factors, or biomaterials in the desired location to achieve more complicated multicell tissue structures[37]. In contrast to cultures in 2D alginate/gelatin hydrogel (Alg-Gel) scaffolds, 3D Alg-Gel scaffolds can be printed in a seven-layer coin shape and loaded with DPSCs. These DPSC-loaded 3D printed scaffolds achieved higher cell proliferation, odontoblastic differentiation, and bone mineralization, suggesting that a 3D environment is more suitable for cell proliferation and differentiation[38]. In addition, Park *et al*[39] designed the printing of DSPCs with VEGF in the central zone and bone morphogenetic protein-2 (BMP-2) in

the peripheral area of the 3D-printed construct to fabricate vascularized bone structures. A cone-shaped scaffold was printed with hydroxyapatite/tricalcium phosphate (HA/TCP) powder that was polymerized by an ultraviolet (UV) photoinitiator. DPSCs and SCAP were mixed with collagen gel and loaded into the 3D printed HA/TCP scaffold for dental pulp regeneration[40]. 3D PCL mesh supplemented with PRP containing various growth factors along with DPSCs was custom printed to fit rat calvarial defects for bone regeneration[27]. PRP containing various growth factors, along with DPSCs, was added to 3D printed PCL mesh for bone regeneration in a rat calvaria defect model[27]. A novel DPSC-loaded bioink containing a mixture of amorphous Mg phosphates and ECM increased the bone density during craniomaxillofacial bone regeneration[41]. With the 3D printing technique, the shape, pore size, and gap size can be precisely controlled to study their microenvironmental effects on cell proliferation and differentiation. Polylactic acid scaffolds (PLASs) were printed in different gap sizes, and it was discovered that smaller gaps in 3D PLASs presented with different cellular orientations[42].

In addition to their osteogenic and odontoblastic potential, the chondrogenic potential of DPSCs has been investigated. Zhang *et al*[43] successfully induced DPSCs to undergo a chondrogenic differentiation process, and their synthesis of sulfated glycosaminoglycans was confirmed. DPSCs formed into 3D pellets were subjected to chondrogenic potential investigation, resulting in the enrichment of collagen I deposition. The content of glycosaminoglycan or collagen type II was not enhanced even with the addition of chondroinductive growth factors, suggesting that the chondrogenic lineage of DPSCs favors differentiation into fibrous cartilage rather than hyaline cartilage[44]. DPSCs, derived from cranial neurons, can differentiate into neuron-like cells for axon regeneration and are potential cell sources for neuron regeneration[45,46]. DPSCs were seeded within chitosan-intercalated montmorillonite/poly(vinyl alcohol) (OMMT/PVA) nanofibrous mesh, and they differentiated into neuron-like cells[47]. A thermosensitive heparin-poloxamer hydrogel with DPSCs and bFGF enhanced motor and sensory functional recovery after spinal cord injury repair[48]. Chitosan scaffolds have been demonstrated to enhance neuronal cell survival and differentiation. Zheng *et al*[49] incorporated bFGF into chitosan scaffolds and found that it promoted DPSC differentiation into neuronal cells but did not affect cell survival. Human adipose microvascular endothelial cells were coseeded in a PLLA/poly(lactic-co-glycolic acids) (PLGA) scaffold with DPSCs to fabricate a prevascularized scaffold, which promoted revascularization, axon regeneration, myelin deposition, and sensory recovery in a rat complete spinal cord transection model[50]. Moreover, DPSCs seeded in Matrigel were able to differentiate into endotheliocytes and pericytes in serum-free culture media and secrete VEGF[51].

SHED

SHED cells, first isolated in 2003, present with positive expression of embryonic stem cell markers, such as OCT4 and NANOG, stage-specific embryonic antigens (SSEA-3 and SSEA-4), and mesenchymal stem cell markers (STRO-1 and CD146)[52-54]. Compared to DPSCs, SHEDs showed higher levels of osteocalcin expression and alkaline phosphatase activity[55]. SHEDs were confirmed to be more immature than DPSCs, allowing them to be "osteoblast-like" and "odontoblast-like", expressing osteocalcin and RUNX-2 markers[53]. Moreover, when SHEDs were cultured in medium with dexamethasone, they differentiated into adipocytes. After *in vitro* culturing for 2 wk in osteogenic medium, extracellular mineralized matrix started to be secreted by the SHEDs. This multilineage potential makes SHEDs alternative sources of dental stem cells[56].

Applications in 3D tissue regeneration

SHEDs cultured *in vitro* for 7 d were found to aggregate together, and they started to form a 3D ossification hemisphere after 36 d[56]. This mineral matrix was identified by alizarin red staining within the self-formed 3D woven bone tissue. SHEDs can be applied in a 3D polylactoglycolide scaffold fabricated by a surface-selective laser sintering device. The expression of osteocalcin was elevated in SHED-loaded polylactoglycolide scaffolds, suggesting that SHEDs are promising cell sources for scaffold populations in tissue bone engineering[57]. In addition to bone regeneration, SHEDs may be a source of neurons. When they were incubated in neurodifferentiation medium supplemented with epidermal growth factor (EGF) and fibroblast growth factor (FGF), SHEDs showed increased expression of neuron markers, such as βIII-

tubulin, microtubule-associated protein 2, tyrosine hydroxylase, and Nestin[58]. These results confirmed the neurogenic potential of SHEDs. In spinal cord injury, a supply of SHEDs rescues hindlimb locomotor function[52]. Furthermore, SHED-conditioned medium was demonstrated to regenerate peripheral nerves in sciatic nerve defects in a rat model. The rat static nerve defects at the mid-thigh level were covered with silicon conduits containing SHED-conditioned medium and resulted in an increase in Schwann cells, axon density and the number of regenerated myelinated fibers[59]. Injection of SHEDs into the brain at the site of perinatal hypoxia-ischemia (HI) injury improved the survival rate of HI-injured mice through inhibition of the expression of proinflammatory cytokines[60]. Although SHEDs have multidifferentiation potency and fewer limitations in terms of ethical concerns in their clinical application, only a few studies have investigated the application of SHEDs in 3D-printed scaffolds for tissue regeneration. It is possible that the collection, treatment methods, and storage of harvested SHEDs have not been standardized or popularized.

PDLSCS

Periodontitis is a very common oral disease resulting in periodontal tissue destruction and, more seriously, tooth loss[61]. Many periodontal regeneration treatments have been performed to restore the damaged periodontium. PDLSCs were isolated from mature periodontal ligaments and found to express the stem cell markers CD105, CD90, and CD73[62-64]. Seo *et al*[63] successfully isolated PDLSCs from human third molars, and the expression of the stem cell markers STRO-1 and CD146/MUC18 was found in PDLSCs. In addition to the expression of stem cell markers, the osteogenic and adipogenic potential of PDLSCs was also identified[65], which makes PDLSCs alternative cell sources for tissue regeneration. The regeneration steps of periodontal tissue were demonstrated by PDLSCs incorporated with hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) as carriers[66]. First, the proliferation of PDLSCs was increased, and collagen matrices were formed. Subsequently, the collagen fibers started to assemble, and cemental-like tissue was observed. Mineralization was present in the cemental-like tissue, and along with the presence of Sharpey's fibers, mature collagen fibers were present. Later, the maturation of cemental-like tissue was identified by the expression of cemental tissue genes, such as α-smooth muscle actin antibody, collagen type XII (ColXII), osteoblast specific factor-2/periostin, and aspirin/PLAP-1[67].

Application in 3D tissue regeneration

A 3D collagen scaffold was fabricated with precise control of the pore size, pore wall alignment, and percolation diameter to investigate the effect of the scaffold structure on periodontal tissue regeneration. The results suggested that a larger percolation diameter increased PDLSC cell elongation and directionality, whereas the pore size influenced cell invasion and cell distribution[68]. In addition to the manipulation of the scaffold structure, the addition of growth factors also promoted the capacity of tissue regeneration. During cemental tissue formation, connective tissue growth factor (CTGF) was found to promote the differentiation of periodontal ligament fibroblasts during the process of osteogenesis[69]. BMP-7, expressed in the cementum, alveolar bone, and periodontal ligament, induces cementogenic differentiation by acting as a progenitor for cementoblasts[70,71]. The expression of BMP-2, localized only in alveolar bone, was also involved in cementogenic differentiation by increasing the expression of cementum attachment protein (CAP)[72]. Since CTGF, BMP-7, and BMP-2 are beneficial for periodontal ligament formation, Cho *et al*[73] compared the effect of these three growth factors by incorporating them into 3D printed PLGA microspheres, and the results indicated that BMP-7 triggered thicker cementum-like layers, better integration with the dentin surface and higher expression of cementum protein 1[73]. In addition to supplying growth factors to promote tissue regeneration, inhibition of inflammatory reactions can also improve tissue formation. For instance, Liu *et al*[74] demonstrated that reductions in tumor necrosis factor-alpha and interferon-gamma levels by the introduction of BMMSCs enhanced bone regeneration. Cao *et al*[75] demonstrated that aspirin promoted BMMSC-based calvarial bone regeneration. Thus, platelet-rich fibrin-containing PDLSCs were treated with aspirin, a non-steroidal anti-inflammatory drug, which increased periodontal bone formation[76].

Instead of providing a direct supply of factors that are required for tissue regeneration, human umbilical vein endothelial cells (HUVECs) were cocultured with PDLSCs to form 3D cell sheet constructs, which were wrapped around human tooth

roots for implantation into the subcutaneous layer of mice. The HUVEC and PDLSC coculture group exhibited the thickest PDL ligament-like arrangement compared to the PDLSC-only group, suggesting that HUVECs contributed to regulating the thickness of the periodontal compartment[77]. Another strategy for improving the supply of vasculature to bone regeneration is the introduction of genetically modified PDLSCs. A lentiviral construct containing platelet-derived growth factor BB (PDGF-BB), an angiogenic gene, was introduced into PDLSCs to overexpress PDGF-BB. A PLGA-PEG-PLGA thermal hydrogel seeded with PDLSCs overexpressing PDGF-BB promoted bone formation in alveolar bone defects[78]. To investigate the possibility of incorporating somatic MSCs in tissue regeneration, a mixture of PDLSCs, somatic MSCs, and DPSCs was cocultured within 3D collagen/chitosan scaffolds for odontogenic differentiation[79]. The results indicated that many growth factors, transcription factors and signaling molecules involved in odontogenic differentiation were significantly promoted in the group mixed with somatic MSCs. In addition to the application of periodontal tissue regeneration, 3D PDLSC-loaded constructs were applied to study the effect of the growth microenvironment on PDLSC differentiation. PDLSCs were seeded in a customized 3D cell-laden hydrogel array with a gradient of gelatin methacrylate (GelMA) and poly(ethylene glycol) (PEG) dimethacrylate compositions to study the response of PDLSCs to ECM[80]. The higher the ratio of PEG was, the better the performance of the PDLSCs in cell proliferation and cell spreading, indicating that the composition of the ECM influenced the behavior of the PDLSCs.

SCAP

SCAP is only present at the tip of the developing tooth root before the tooth erupts. Although SCAP shares some similar characteristics with DPSCs, there are still some differences between these two types of stem cells[8]. In contrast to DPSCs, which are the sources of replacement odontoblasts, SCAP is the primary source of odontoblasts involved in the formation of root dentin[81]. Comparing their *in vitro* osteo/odontogenic differentiation potential with DPSCs, SCAP presents stem cell markers (STRO-1, CD146, and CD34) similar to those of DPSCs but with a significantly higher proliferation rate and mineralization potential during dental formation[82]. Other MSC markers, CD73, CD90, and CD105, were also identified in SCAP[40]. Liu *et al*[83] found that CD24 was exclusively expressed in SCAP, not in DPSCs. SCAP are comparatively easy to isolate from the tips of developing roots. They are digested with a cocktail of collagenase to isolate single-cell suspensions, which are grown under routine cell culture conditions[84].

Application in 3D tissue regeneration

In addition to using residual dental pulp in dentin regeneration, SCAP with osteogenic potential obtained from dental roots have been applied for dentin regeneration[85]. Injectable PLLA nanofibrous microspheres (NF-MS) with the ability to controllably release BMP-2 were encapsulated in SCAP for dentin regeneration[86]. More mineralization and osteodentin formation were observed in NF-MS with controlled BMP-2 release microspheres, suggesting their potential for dental tissue repair. In addition to BMP-2 release, SCAP cotreated with stromal cell-derived factor-1α, which is able to promote odontoblast differentiation of dental pulp cells, were shown to undergo odontogenic differentiation-related gene and protein expression[87]. PDGF-BB is known to promote angiogenesis during tissue regeneration[88,89]. The addition of PDGF-BB promoted the proliferation of SCAP and improved new bone formation and mineralization in a rat calvaria defect model[90].

The growth factor TGBβ3 was shown to be involved in tissue regeneration[91]. Somoza *et al*[92] observed that TGBβ3 secretion by SCAP was elevated when they were grown in a 3D microenvironment regardless of the materials used for the scaffold. Thus, SCAP were applied and incorporated into a 3D scaffold for tissue regeneration. Considering the secretion properties of SCAP, Na *et al*[93] developed a 3D scaffold-free stem-cell sheet-derived pellet (CSDP) by culturing a large amount of SCAP on a culture dish to form a cell sheet that enriched the secreted ECM. CSDP exhibited the odontogenic/osteogenic potential to form dental pulp-like and dentine-like tissue after implantation into the subcutaneous layer in immunodeficient mice. Dental ECM was reported to enhance cell proliferation and mineralization[94]. A novel SCAP-loaded bioink was developed by applying dental ECM to printable alginate to form dentin-derived bioink, in which soluble dentin molecules significantly enhanced odontogenic

differentiation[95].

DFPCS

The dental follicle is the connective tissue surrounding the enamel organ and dental papilla that forms a vascular fibrous sac. In 2005, Morsczeck *et al*[96] isolated DFPCs from the dental follicle of human third molar teeth, which were found to express the stem cell markers Notch and Nestin. Their potential for osteogenic, adipogenic, chondrogenic, and neural differentiation was further confirmed[97]. Subsequently, DFPCs were applied for tissue regeneration, such as the regeneration of the salivary glands, dental roots, and bone tissue[98-100].

Application in 3D tissue regeneration

Among the applications of dental stem cells in tissue regeneration, only a few studies have introduced DFPCs to 3D tissue regeneration. DFPCs cultured in a 3D rotatory culture system displayed many follicle markers, such as CD44, CD90, CD146, CD31, CD34, and CD45Ag[101]. Furthermore, their differentiation potential was increased when DFPCs were cultured in a 3D dynamic culture system. For the generation of 3D tissue constructs, DFPCs were seeded in 3D porous scaffolds of collagen-nanohydroxyapatite/phosphoserine (collagen-nano-HA/OPS) biocomposite cryogels and implanted into the subcutaneous layer of *nu* mice. These 3D DFPC-loaded collagen-nano-HA/OPS constructs exhibited greater osteogenic differentiation with higher levels of osteopontin secretion[102].

CLINICAL APPLICATIONS OF DENTAL STEM CELLS

The use of dental stem cells for autologous or allogeneic transplantation has been introduced into clinical practice. The biological safety of dental stem cells requires strict regulation. Standard examinations for viruses, pathogenic microorganisms, or any sources with animal origins are necessary[103-105]. Due to the immune response, a same-species origin of the stem cell culture system is recommended for cell therapy [106]. According to the Clinical Gov website, there are fewer than 10 cases of the use of dental stem cells in clinical applications, implying a gap in the application of dental stem cells between basic research and clinical practice. There is a scarcity of data for the use of decellularized biological membranes for preparing 3D dental regenerative constructs, which is a crucial approach for regenerative dentistry. Indeed, dental stem cells are not the most suitable stem cell choice for tissue regeneration due to harvest contamination, small cell amounts available per patient and invasive harvesting approaches. However, the regenerative potential of dental stem cells is still supported by several clinical results. A clinical study reported that most clinical trials based on the use of DPSCs cells were performed for bone regeneration, periodontitis, and dental pulp regeneration, whereas trials involving the use of periodontal PDLSCs were conducted to study periodontal disease treatment. No clinical trials that used DFPCs were found[107]. Overall, dental stem cells are not commonly used to treat human diseases. Identical to the original issues hindering stem cell therapy, ethical concerns and cell sources are the main obstacles. Moreover, the survival of grafted dental stem cells exhibited different results after long-term follow-up observations. Autologous PDLSCs were detected after 8 wk in an ovine periodontal defect model, whereas donor PDLSCs implanted into recipient mice were untraceable two weeks after implantation [108]. Whether autologous or allogeneic stem cell sources affect the survival rate of transplanted cells remains to be further investigated.

CONCLUSION

Dental-derived stem cells with mesenchymal stem cell properties are promising cell sources for tissue regeneration. Comparisons among these five types of dental-derived stem cells showed that DPSCs, SHEDs, and PDLSCs present a higher growth potential than BMSCs[109]. Moreover, SCAP and DPSCs showed weaker adipogenic differentiation than BMSCs[84]. Regardless of whether the different types of dental stem cells have osteogenic or odontogenic potential, each cell type presents unique differentiation potentials in the corresponding tissue type. Although dental stem cells present

differentiation potential for adipogenesis, chondrogenesis, and neurogenesis, most of their clinical utility lies in the field of regenerative dentistry. With the trend of 3D tissue engineering, the application of dental stem cells to 3D tissue reconstruction has been emphasized. In this review, many basic research and preclinical studies were presented to support the idea that dental stem cells can be applied in a feasible approach to translational medicine and are available resources for 3D tissue regeneration.

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REVIEW

Priming strategies for controlling stem cell fate: Applications and challenges in dental tissue regeneration

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Abstract

Mesenchymal stromal cells (MSCs) have attracted intense interest in the field of dental tissue regeneration. Dental tissue is a popular source of MSCs because MSCs can be obtained with minimally invasive procedures. MSCs possess distinct inherent properties of self-renewal, immunomodulation, proangiogenic potential, and multilineage potency, as well as being readily available and easy to culture. However, major issues, including poor engraftment and low survival rates *in vivo*, remain to be resolved before large-scale application is feasible in clinical treatments. Thus, some recent investigations have sought ways to optimize MSC functions *in vitro* and *in vivo*. Currently, priming culture conditions, pretreatment with mechanical and physical stimuli, preconditioning with cytokines and growth factors, and genetic modification of MSCs are considered to be the main strategies; all of which could contribute to improving MSC efficacy in dental regenerative medicine. Research in this field has made tremendous progress and continues to gather interest and stimulate innovation. In this review, we summarize the priming approaches for enhancing the intrinsic biological properties of MSCs such as migration, antiapoptotic effect, proangiogenic potential, and regenerative properties. Challenges in current approaches associated with MSC modification and possible future solutions are also indicated. We aim to outline the present understanding of priming approaches to improve the therapeutic effects of MSCs on dental tissue regeneration.

Key Words: Mesenchymal stem cells; Priming; Dental regeneration; Culture conditions;

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Core Tip: Undoubtedly, the efficacy associated with the survival and regenerative properties of unmodified mesenchymal stromal cells (MSCs) cannot be overemphasized. These properties cannot be augmented until these cells are enhanced by priming approaches to protect MSCs against an inhospitable microenvironment *in vivo*. In this review, we focus on discussing the current MSC priming approaches in the field of dental tissue regeneration. As a promising outcome, primed MSCs can exhibit prolonged therapeutic efficacy and can be applied to the clinical treatment of some dental diseases in the near future.

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INTRODUCTION

Research on mesenchymal stromal cell (MSC)-based therapy has made rapid strides over recent decades due to the beneficial biological effects of these cells. MSCs, which are also known as mesenchymal stem cells, are spindle-shaped cells located at perivascular sites in various human tissues and organs, including bone marrow, adipose tissue, umbilical cord, and dental tissue. Each of these MSC sources has its own advantages and disadvantages[1]. In general, MSCs are readily available and easy to culture *in vitro*, with genetic stability. MSCs can be characterized based on their specific properties: adherence to plastic and a typical immunophenotypic profile (expression of the surface markers CD44, CD73, CD90 and CD105, and a lack of CD34, CD45, CD14 and HLA-DR)[2]. MSCs possess multilineage-differentiation potential into osteoblasts, chondrocytes, adipocytes, and even highly specialized cells, such as myoblasts[3], neurons[4], endothelial cells[5], and hepatocytes[6,7]. In addition, the low immunogenicity and outstanding immunomodulatory properties of MSCs make them ideal therapeutic cell candidates[8]. To date, the experimental and preclinical applications of MSCs span various diseases and conditions, accompanied by promising outcomes.

Over the last few decades, the search for MSC-like cells in specific tissues has led to the discovery of distinct populations of MSCs from various human dental tissues. Dental tissue is a popular MSC source. Compared to cells from other tissues, MSCs from dental tissue can be obtained through minimally invasive procedures. Currently, five main populations of dental tissue-derived MSCs have been successfully isolated and characterized. Postnatal dental pulp stem cells (DPSCs)[9] were the first human dental MSCs identified in pulp tissue. Later, other types of dental tissue-derived MSCs were gradually discovered, including stem cells from human exfoliated deciduous teeth (SHEDs)[10], periodontal ligament stem cells (PDLSCs)[11], dental follicle precursor cells (DFCs)[12] and stem cells from the apical papilla (SCAPs)[13]. In dental tissue, these five types of MSCs are accessible MSC-like populations with superior self-renewal capacities, immunomodulatory functions, and multilineage-differentiation potential for tissue regeneration.

Nevertheless, the beneficial effects of MSC-based regeneration are not always fulfilled. MSC properties can be influenced by *in vitro* and *in vivo* biological, biochemical and biophysical factors *via* reciprocal cell-to-cell interactions, the extracellular matrix (ECM), and soluble bioactive factors[14]. MSCs *in vivo* interact with surrounding cells and tissues in a three-dimensional (3D) microenvironment, producing anti-inflammatory molecules, promoting angiogenesis, preventing cell death, and reconfiguring the ECM[15]. Moreover, MSCs reside in a microenvironment with relatively low oxygen tension (*i.e.*, 1%–5% O_2) *in vivo*, while the O_2 tension *in vitro* (*i.e.*, 20%–21%) is much higher than in the original MSC microenvironment[16]. The

different O_2 tension associated with *in vitro* culture can decrease cell-regenerative capacities, including proliferation, differentiation, and anti-inflammatory responses. MSCs are generally cultured in nutrient- and O_2-enriched environments *in vitro*. In contrast, transplanted MSCs are confronted with harsh conditions such as lack of blood supply and inflammation induced by tissue damage; both of which cause apoptosis and senescence, leading to the failure of regeneration. In this context, MSCs have shown promising regenerative properties for tissue repair; however, exploring more effective strategies is still necessary to improve their therapeutic efficacy.

MSC properties cannot be augmented without further enhancement through by priming approaches to protect MSCs against an inhospitable microenvironment *in vivo* . To this end, some recent investigations have sought ways to improve MSC functions *in vitro* and *in vivo*. Optimizing culture conditions, preconditioning with cytokines and growth factors, and genetic modifications of MSCs are considered to be the main strategies; all of which contribute to improving MSC transplantation efficiency for tissue regeneration[17]. Several studies have revealed that pretreated MSCs exhibit better cell survival, augmented homing abilities to injury sites, enhanced immunomodulatory properties, optimized proangiogenic abilities, and increased multilineage-differentiation capabilities[18-20]. Figure 1 shows the six focal improvements in MSCs that contribute to improved therapeutic effects. Research in this field has made tremendous progress and continues to gather interest and spur innovation. In this review, we summarize the approaches proposed to improve dental tissue-derived MSC functions for dental tissue regeneration and divide these approaches into four categories: (1) Culture condition manipulation; (2) Pretreatment with mechanical and physical stimuli; (3) Preconditioning with growth factors and cytokines; and (4) Gene modification. We further discuss these approaches mainly in the context of enhancing the intrinsic biological properties of MSCs such as migration, anti-apoptotic effect, immunomodulation, proangiogenic potential, and regenerative properties.

DENTAL TISSUE-DERIVED MSCS

Characteristics of dental MSCs

DPSCs are MSC-like cells in the pulp chamber of permanent teeth that originate from the cranial neural crest. Figure 2 shows the different dental regions from which DPSCs originate, together with other dental tissue-derived MSCs. Similar to bone-marrow-derived MSCs, DPSCs have enriched expression of the surface markers Stro-1, CD29, CD73, CD90, CD105 and CD166, while they are negative for hematopoietic markers such as CD14, CD45, CD34, CD25 and CD28[9]. DPSCs exhibit rapid proliferation and superior immunosuppressive properties, and they are prone to forming dentin/pulp-like complexes. Aside from their odontogenic potential, DPSCs can be reprogrammed into adipocytes, chondrocytes, myocytes and neural cells[21].

SHEDs were isolated from the dental pulp tissue of exfoliated deciduous teeth. SHEDs share similar MSC regenerative capacities such as self-renewal and multilineage differentiation potential[10]. However, these cells exhibit increased proliferation rates and the spontaneous formation of sphere-like cell clusters. SHEDs are distinct from DPSCs[22]. In addition to the expression of DPSC surface markers, SHEDs also highly express the embryonic stem cell markers Oct4 and Nanog, stage-specific embryonic antigen (SSEA)-3 and SSEA-4, and the neural stem cell marker nestin. After the induction of neurogenesis, SHEDs show higher expression than DPSCs of neuronal and glial cell markers, such as β-III-tubulin, tyrosine hydroxylase, microtubule-associated protein (MAP)2, and nestin[23].

SCAPs and DFCs are MSCs that are derived only from developing permanent teeth. SCAPs are found at the apices of growing teeth, and DFCs are located in connective tissue sacs surrounding the enamel organ[13,24]. These two types of dental MSCs can form adherent clonogenic clusters and differentiate into adipocytes, odontoblasts/osteoblasts, cementoblasts, and periodontal ligament. SCAPs have been reported to possess greater potential to form dentin than DPSCs, due to their higher proliferation capacity and greater telomerase activity. DFCs are regarded as the parent cells of periodontal tissue and can form periodontal tissues, including alveolar bone, periodontal ligament, and cementum.

PDLSCs are derived from the human periodontal ligament, which is a connective tissue that lies between the cementum and the alveolar bone socket. PDLSCs have been demonstrated to be a reliable source of periodontal tissue regeneration. Compared to DPSCs, PDLSCs exhibit higher expression of scleraxis, a tendon-specific transcription factor[11]. These cells can be readily expanded *in vitro* and generate

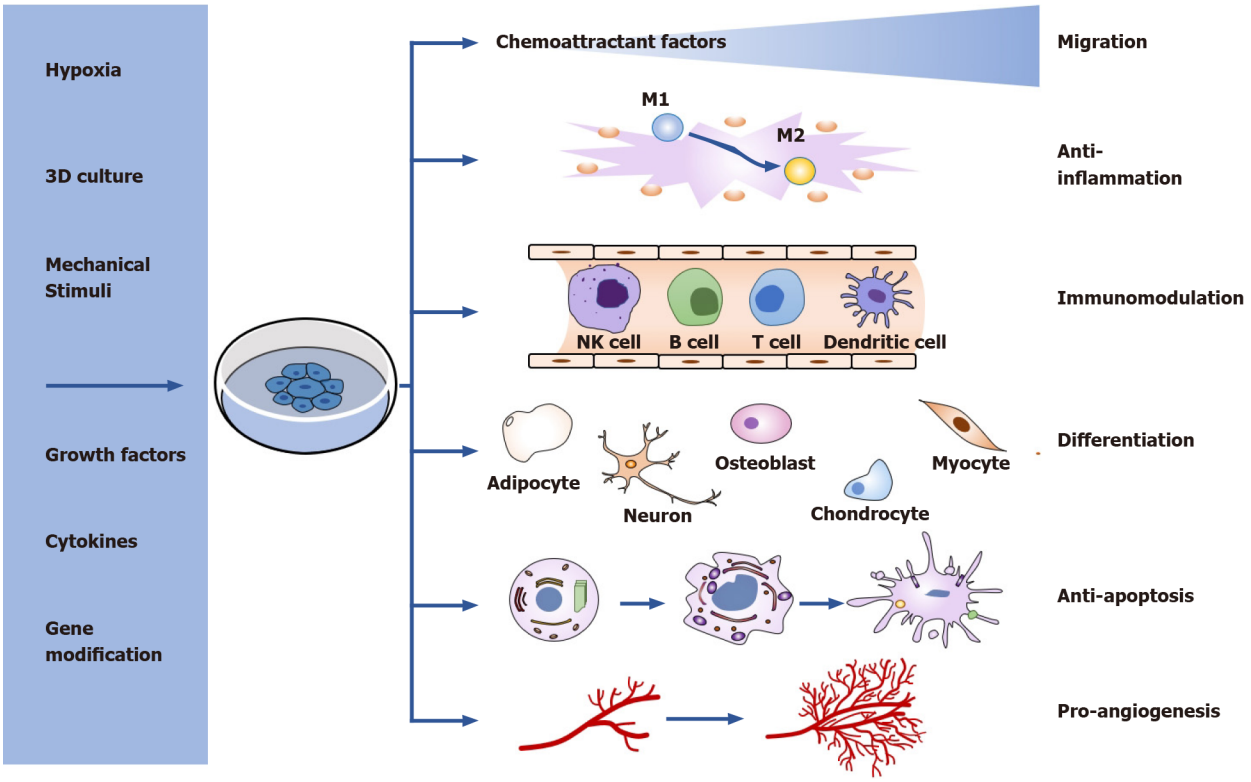

Figure 1 Overview of the functional improvements of mesenchymal stromal cell (MSC) properties by priming strategies. MSCs can be primed *via* different approaches, such as priming culture conditions, pretreatment with mechanical and physical stimuli, preconditioning with cytokines and growth factors, and genetic modification. As promising outcome, primed MSCs can exhibit prolonged therapeutic efficacy, including migration, anti-apoptosis, anti-inflammation, immunomodulation, proangiogenesis, and regenerative properties. The improved capabilities are interconnected and greatly influenced by priming approaches.

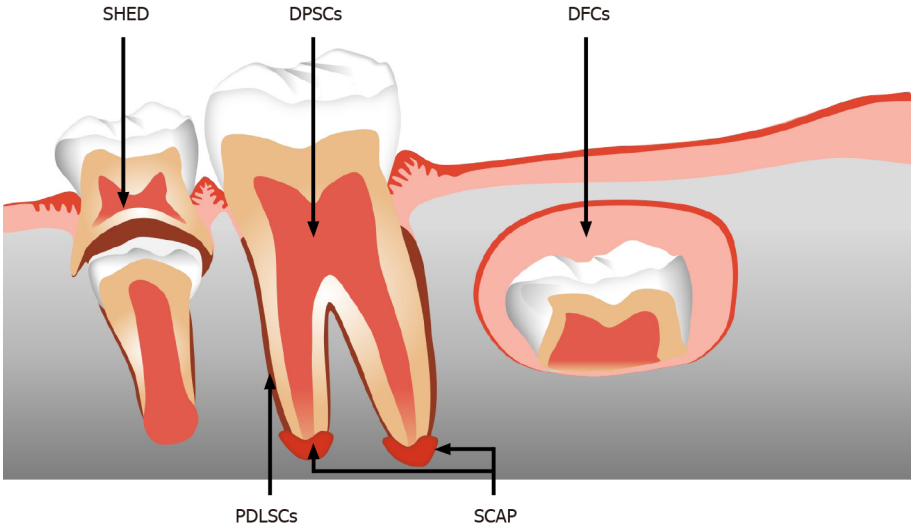

Figure 2 Schematic image of dental mesenchymal stromal cells from different tissue regions. SHED: Stem cells from exfoliated deciduous teeth; DPSCs: Dental pulp stem cells; DFCs: Dental follicle precursor cells; PDLSCs: Periodontal ligament stem cells; SCAP: Stem cells from apical papilla.

cementum/periodontal ligament-like complexes *in vivo*. In addition, the osteogenic differentiation capability of PDLSCs was demonstrated by the formation of calcified nodules and the expression of alkaline phosphatase (ALP), matrix extracellular protein (MEPE), bone sialoprotein (BSP), osteocalcin (OCN), and transforming growth factor (TGF)-β receptor I.

Dental MSC applications and limitations

Dental MSCs have been extensively investigated in preclinical studies. Moreover,

several clinical trials have been reported in recent years[25-27]. Some reviews have already summarized the benefits of dental MSCs in regenerative medicine[28-30]. In short, dental MSCs have been reported to promote the regeneration of dental tissues, bone, cartilage, muscle, and nerves[31-34]. Moreover, dental MSCs have also been implicated in the treatment of various diseases, such as brain ischemia[35], liver fibrosis[36], diabetes[37], rheumatoid arthritis[38], and Alzheimer's disease[39]. However, dental MSCs have thus far exhibited only moderate benefits in clinical studies, and researchers are still struggling to move forward to advanced phases (III and IV) of clinical trials. To secure efficient and successful translation of clinical procedure for dental MSCs, substantial approaches concerning functional improvements must be established.

CELL FUNCTION OPTIMIZATION STRATEGIES

Culture condition manipulation

In general, MSCs reside in a confined microenvironment called the stem cell niche *in vivo*, which includes not only MSCs themselves but also other supporting cells and ECM. The stem cell niche is the basis of tissue homeostasis. Dental MSCs are isolated from individuals; these cells must be cultured and expanded *in vitro* to obtain a sufficient number of cells before transplantation. However, *in vitro* culture does not entirely replicate *in vivo* cell behavior, and cells may lose their tissue-specific functions. Cell culture conditions, such as O_2 tension and 3D culture, influence cell behavior. Recreating the physical and mechanical microenvironment experienced by MSCs *in vivo* is important in reproducing the stem cell niche. Several studies have reported that manipulation of conventional culture conditions could enhance the regenerative efficacy of MSCs. Here, we introduce two widely used alternative culture methods: hypoxic preconditioning and 3D spheroid culture. The enhancement of intrinsic biological properties of MSCs by culture condition manipulation and other priming strategies are summarized in Table 1.

Hypoxic preconditioning

O_2 is one of the critical factors associated with cellular homeostasis, as a lack of O_2 could result in disease pathogenesis. O_2 tension varies from 1% to 12% in the physiological state in adult organs and tissue, depending on the degree of tissue vascularization and metabolic activities[16,40]. MSCs residing in the general dental microenvironment are exposed to low O_2 tension (*i.e.*, 3%–6% O_2)[41], while MSCs *in vitro* are typically exposed to higher O_2 concentrations (*i.e.*, 20%–21% O_2). The negative impact of ambient O_2 tension on MSCs cultured *in vitro*, such as decreased proliferative capacity, DNA damage, and senescence, was reported by a number of studies [42-46]. When MSCs are expanded in normoxia and then transplanted into injured tissue, they face hypoxic conditions and undergo apoptosis. Hypoxic preconditioning of MSCs is regarded as a better way to mimic the naïve MSC niche and improve their therapeutic potential than normoxic culture conditions[47].

In general, hypoxic conditions require a reliable experimental device to maintain stable O_2 tension for cell culture. Commonly used CO_2 incubators have difficulty producing low O_2 levels. Currently, there are several approaches to achieve hypoxic conditions for cultured cells[48]. One approach involves using a specialized hypoxic chamber inside a standard CO_2 incubator. This is a convenient and low-cost method. However, the major drawback is gas leakage that may disrupt the experimental processes and cause fluctuations in O_2 concentrations in the incubator. Moreover, O_2 concentrations can be temporarily disturbed by every time incubator doors are opened and take time to stabilize. Hypoxic culture can also be performed in a tri-gas incubator, which is an effective device to generate stable experimental O_2 concentrations. CO_2 and nitrogen (N_2) are supplied to reduce the O_2 levels in the incubator. Because of ability to control the gas mixture (CO_2, O_2 and N_2), the tri-gas incubator is currently considered to be a practical approach to provide the closest conditions to those in the body. A third method is pharmacological agents that stimulate hypoxia. Cobalt chloride ($CoCl_2$)[49,50] and deferoxamine (DFO)[51] are well-known hypoxia mimetics that act by inducing the expression of hypoxia inducible factors (HIFs), which play vital roles in the hypoxia signaling pathway and guide the cellular response to hypoxia. Stabilization of HIF-1 can also be achieved by propyl hydroxylase inhibitors (PHDs), which block enzymatic activity to inhibit HIF-1 degradation during hypoxia[52].

Table 1 Priming strategies for controlling mesenchymal stromal cell fate

Priming strategies		Cell type	Benefits	Mechanisms	Reference
Culture conditions	**Hypoxia:**				
	1%–3% O_2	DPSCs; PDLSCs	Improve survival	Upregulation of stem cell markers; Regulation of metabolic activities; Activation of the p38/MAPK and ERK/MAPK pathways	[66,67,70]
		DPSCs; SHEDs; SCAPs	Promote angiogenesis	Increase proangiogenic factors releasing	[60,62,63]
		DPSCs; SCAPs; PDLSCs	Enhance differentiation potential	Upregulation of odontoblastic markers	[68,69,71,72]
		PDLSCs	Enhance anti-inflammation effect	Upregulation of IL-37	[73]
	Pharmacological stimulation	DPSCs	Promote angiogenesis	Increase intracellular levels of HIF-1α	[51,64,65]
		PDLSCs	Improve survival but inhibit differentiation potential		[49]
	3D culture:				
	Single cell type	DPSCs; PDLSCs	Enhance differentiation potential	Upregulation of odontoblastic markers	[83,84,87]
	Coculture	DPSCs and ECs	Promote angiogenesis		[86]
	Mechanical and physical stimuli:				
	LIPUS	DPSCs; PDLSCs	Increase proliferation	Activation of MAPK pathway	[94,95]
	Cyclic mechanical tension	DPSCs	Promote osteogenic differentiation; Increase cytokines release	Upregulation of osteoblastic markers	[98,99]
	Uniaxial stretch	DPSCs	Increase proliferation but inhibit osteo/odontogenic differentiation		[96,97]
	Cytokines				
	SDF-1	DPSCs; PDLSCs	Promote cell migration	Activation of SDF-1/CXCR4 axis; Autophagy; Activation of AKT and GSK3β/β-catenin pathways	[104,107,109,110]
		PDLSCs	Anti-apoptosis	Activation of ERK pathway	[105]
		DPSCs; PDLSCs	Enhance differentiation potential	Upregulation of odontoblastic markers; Upregulation of osteoblastic markers	[106,108,111]
	TNF-α		Enhance immunomodulatory effects	Mediated by TNF/TNFR2 signaling	[124-126]
			Enhance osteogenic differentiation	Activation of p38 pathway; Activation of miR-21/STAT3 and NF-κB pathway	[127-129]
			Inhibit differentiation potential (50-100 ng/mL)	Activation Wnt/β-catenin pathway	[132]
	G-CSF; IFN-γ	DPSCs	Promote cell migration		[114,121,122]
		DPSCs	Enhance or inhibit differentiation potential depend on cytokines concentration		[116,120,121]
Preconditioning mediators	**growth factors**				
	bFGF	DPSCs	Promote angiogenesis		[136,139-141]
		DPSCs; PDLSCs	Enhance differentiation potential on dose dependent (20-50 ng/mL in vitro; 15 μg/mL-5 mg/mL in vivo)	Upregulation of odontoblastic markers; Upregulation of osteoblastic markers; Upregulation of neural markers; Activation of FGFR/MEK/ERK1/2 and BMP/BMPR signaling pathways	[137,138,141,144,149-151]

		DPSCs	Promote anti-inflammation effect	Altered cytokines expression;	[146-148]
	IGF-1	PDLSCs	Promote cell survival		[165]
		DPSCs	Anti-apoptosis		[164]
		DPSCs; PDLSCs	Enhance differentiation potential	Upregulation of osteoblastic markers; Upregulation of odontoblastic markers; Activation of mTOR pathway; Target of EphrinB1	[160-162,166]
	Sox-2	DPSCs	Improve cell migration		[171]
	Bcl-2; Oct-4	DPSCs	Improve cell survival	Upregulation of stemness-rated genes;	[168,169]
Genetic modification	Foxo-1	PDLSCs	Promote anti-inflammation effect	Resistance to oxidative stress	[175]
	BMP family; Runx2	DPSCs; SCAP; DFCs	Enhanced differentiation potential	Upregulation of osteoblastic markers; Upregulation of odontoblastic markers	[178-184]

DPSCs: Dental pulp stem cells; PDLSCs: Periodontal ligament stem cells; SHED: Stem cells from human exfoliated deciduous teeth; SCAP: Stem cells from the apical papilla; LIPUS: Low-intensity pulsed ultrasound; SDF-1: Stromal cell-derived factor-1; TNF-α: Tumor necrosis factor-α; G-CSF: Granulocyte-colony stimulating factor; IFN-γ: Interferon-γ; bFGF: Basic fibroblast growth factor; IGF-1: Insulin-like growth factor-1; Bcl-2: B-cell lymphoma 2.

Hypoxic preconditioning was reported to have positive impacts on the survival and proangiogenic properties of MSCs. When cultured in low-serum medium, 1% O_2 pretreated MSCs can prevent damage through increased paracrine secretion of proangiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)[53]. Low O_2 concentrations also increased metabolic activity and decreased caspase-3/7 activity, thus reducing the sensitivity of MSCs to the ischemic microenvironment[54]. MSCs induced by 2% O_2 showed decreased tumorigenic potential, as indicated by significantly reduced transformation into tumor-associated fibroblasts[55]. Compared to normoxic conditions at 20% O_2, hypoxic conditions at 3% O_2 improved genetic and chromosomal stability, ensuring the safety of MSCs[56]. Regarding MSCs derived from dental tissue, hypoxic preconditioning has been evaluated in DPSCs[57,58], SHEDs[59], SCAPs[60] and PDLSCs[61].

DPSCs, SHEDs and SCAPs are involved in pulp regeneration. These cells have been demonstrated to support the process of pulp revascularization under hypoxia by releasing proangiogenic molecules[60,62,63]. DFO, $CoCl_2$, and PHD inhibitors induce hypoxia-stimulated VEGF production in explanted dental pulp by increasing intracellular levels of HIF-1α[51,64,65]. The mesenchymal stem cell marker STRO-1 is reported to be increased in dental pulp cells under hypoxic conditions[66]. Fukuyama *et al*[67] revealed that the proliferation of dental pulp cells was initially suppressed under hypoxia but increased afterward because of activation of the metabolism-related enzyme AMP-activated protein kinase. Hypoxic conditions can modulate the mineralization potential of DPSCs. Higher expression of odontoblastic markers such as OCN, dentin sialophosphoprotein (DSPP), and dentin matrix acidic phosphoprotein-1 (DMP1) was observed under hypoxia[68]. SCAPs preconditioned with 1% O_2 exhibited upregulated osteogenic and neuronal differentiation as well as angiogenesis[69].

Hypoxia promoted PDLSC clone formation and proliferation *via* the p38/MAPK and ERK/MAPK signaling pathways[70]. A study on PDLSCs under hypoxia showed enhanced osteogenic differentiation both *in vitro* and *in vivo*[71]. This effect could be the reason that hypoxia mediates the expression of RUNX2 in PDLSCs *via* HIF-1α-induced VEGF[72]. However, hypoxia induced by $CoCl_2$ maintained the stemness of PDLSCs while inhibiting the osteoblastic differentiation of PDLSCs[49]. The secretome of hypoxia-preconditioned PDLSCs showed an augmented anti-inflammatory effect mediated by interleukin (IL)-37 expression[73]. After culture in 1% O_2 for 24 h, the proteomic profile of PDLSCs, mainly proteins related to energy metabolism, autophagy, and stimuli-responsive proteins, was changed[74].

These findings highlight the advantages of hypoxic pretreatment in dental MSC culture. However, to enable the practical use of hypoxia-treated dental MSCs, some issues should be considered. In experimental settings, a wide range of O_2 concentrations (from 5% to < 1%) were used in different studies, and each concentration may stimulate different properties of dental MSCs. This issue was indicated in adipose-derived MSCs (ASCs). Choi *et al*[75] reported that the differentiation potential of ASCs was improved significantly by 2%–5% O_2, while a lower O_2 level reduced the effect. Moreover, MSCs of different dental origins require distinct O_2 tensions to meet tissue-

specific demands. For instance, the O_2 concentration required by DPSCs for pulp regeneration could differ from that of PDLSCs for periodontal tissue regeneration. Therefore, the optimal O_2 level for maximizing the capacity of each type of dental MSCs should be determined before clinical application.

3D spheroid culture

In traditional two-dimensional (2D) culture, cells attach to a plastic surface and grow as a monolayer in a culture flask. However, this is a highly artificial environment that fails to recapitulate cell-to-cell or cell-to-ECM interactions, stimulating dedifferentiation capacity and decreasing the therapeutic performance of MSCs[76]. 3D spheroid culture techniques have been applied to overcome this tissue. 3D spherical cell aggregates are multicellular structures that consist of cells, ECM, and paracrine factors, mimicking the spontaneous metabolic microenvironment in both nutrient and O_2 concentrations and providing superior *in vivo* models compared to monolayer culture systems[77]. Several studies have compared the therapeutic effects of 2D- and 3D-cultured MSCs and shown the improved regenerative capacity of spheroid-cultured MSCs[78]. It has been reported that spheroid culture potentiates the proliferative and differentiative capacity of MSCs[79]. MSCs cultured as spheroids showed altered adhesion molecule gene expression patterns and enhanced immunomodulatory capacity[80]. In addition, the augmented migration and homing efficiency of MSCs to the damaged site were found to promote engraftment after *in vivo* transplantation[81].

Based on these findings, the regenerative potential of 3D spheroid cultured dental MSCs was evaluated in various preclinical studies. Xiao *et al*[82] found that DPSCs were able to form large aggregates on Matrigel under osteogenic induction by undergoing a process of central cell death, cavitation, and spontaneous multilineage differentiation. Yamamoto *et al*[83] fabricated 3D DPSC aggregates through a low-attachment method. The DPSC spheroids exhibited improved odonto/osteoblastic differentiation ability *in vitro* that was mediated by integrin signaling. Lee *et al*[84] then demonstrated that 3D DPSC spheres exhibited enhanced odontogenic differentiation *in vivo*. In addition, the spheres were enriched in pluripotency transcription factors, such as Sox2, Lin28, Esrrb and Klf4. 2D- and 3D-cultured DPSCs were further compared by microarray analysis, and the expression of genes related to the ECM, cell differentiation, cell-to-cell/cell-to-matrix, and osteoblast differentiation was promoted [85]. Dissanayaka *et al*[86] fabricated microtissue spheroids by coculturing DPSCs and human umbilical vein endothelial cells in an agarose 3D Petri dish. Upon subcutaneous implantation *in vivo*, effective pulp-like tissue formation and capillary-like structures were successfully anastomosed with the host vasculature. PDLSC spheroids were also examined and showed enhanced osteogenic differentiation regulated by SFRP3-mediated ALP activation[87]. Collectively, DPSC or PDLSC spheroids could maintain their enhanced therapeutic functions both *in vitro* and *in vivo*.

Although there is a general consensus that 3D spheroid culture exhibits therapeutic advantages over monolayer culture, several technical points still need to be considered to optimize 3D culture methods. Currently, hanging drops and low attachment surfaces are the two primary methods of spheroid fabrication, but both methods are inefficient and have low yields in the laboratory. Automated 3D-bioreactor systems have been designed to minimize labor-intensive, time-consuming procedures and improve the yield of 3D-cultured MSCs[76,77,88]. The size and cell number of each spheroid and the culture period are other important factors to consider. Since the core area of spheroids is often hypoxic and lacks nutrients, excessively large spheroids could lead to cell death or dysfunction[89]. In addition, the culture duration (short-term or long-term) tends to affect the density of spheroids, which influences the cell gene expression profiles and other secretory factors[81]. Therefore, researchers need to determine an effective culture protocol to obtain a sufficient number of homogeneous spheroids with stable therapeutic efficacy.

Mechanical and physical stimuli

Dental MSCs are mechanosensitive cells that can recognize and transform mechanical changes into cellular responses[90,91]. Sübay *et al*[92] found that the application of orthodontic extrusive forces to teeth had no significant adverse effect on human pulp tissue. Since then, several studies have shown that mechanical stimuli such as low-intensity pulsed ultrasound (LIPUS), uniaxial mechanical stretch, cyclic mechanical tension and cyclic uniaxial compressive stress are able to induce the proliferation of dental MSCs. In addition, physical stimuli including surface topographies, dynamic hydrostatic pressure and pulsating fluid flow have been reported to promote the differentiation of dental MSCs[93].

The positive effects of mechanical and physical stimuli on the biological behavior of dental MSCs have been well described. Gao *et al*[94] showed that LIPUS, which is applied clinically to promote healing, can promote DPSC, PDLSC and BMMSC proliferation in an intensity- and cell-specific dependent manner *via* activation of distinct mitogen-activated protein kinase (MAPK) pathways. The author further demonstrated that Piezo1 and Piezo2, two mechanosensitive membrane ion channels, contributed to transducing ultrasound-associated mechanical signals and activating downstream MAPK signaling processes in dental MSCs[95]. Two other studies noted that uniaxial stretch increased the proliferation of DPSCs but inhibited their osteo/odontogenic differentiation potential, indicating that additional studies are required to clarify the intra- and intercellular mechanisms associated with mechanical stress[96,97]. To better understand the effect of mechanical stress on dental MSC differentiation, Han *et al*[98] observed increased proliferation and mRNA expression levels of osteogenic markers under cyclic mechanical tension. These findings suggest that the mechanical cyclic tension is a potent positive modulator of osteogenic differentiation in DPSCs. Yang *et al*[99] demonstrated that compressive stress can induce cell morphology changes and odontogenic differentiation in DPSCs. The study showed increased expression of ALP, DMP1, BMP2, DSPP and collagen (COL) type I under compressive stress, indicating that mechanical stimuli could initiate repair mechanisms in the dentin-pulp complex. It should be noted that Lee *et al*[100] showed that mechanical stimuli increased the release of proinflammatory cytokines and antioxidant defense enzymes. This study demonstrated that proinflammatory cytokines and reactive oxygen species produced in response to mechanical strain might play a key role in activating the early cellular signals involved in Nrf2-ARE-mediated gene transcription, providing a guidance for cellular protection or suppressing harmful side effects.

Cytokine and growth factor priming to enhance MSC functions

It is generally accepted that cytokine and growth factor priming may influence host tissues *via* paracrine effects. The interaction between growth factors and their receptors on cell surfaces activates downstream signal transduction for cell survival, migration and differentiation. Moreover, MSCs have inherent immunomodulatory characteristics to inhibit T cell and B cell activation and dendritic cell differentiation, impair the cytolytic potential of natural killer (NK) cells, and promote regulatory T cell (Treg) differentiation[101]. However, the immunomodulatory effect of MSCs is not always achieved but requires stimulation by inflammatory factors, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IL-1β[102]. Therefore, priming MSCs with cytokines and growth factors in this context is suggested to be a supplemental molecular strategy to foster the therapeutic potential of MSCs and contribute to establishing a hospitable microenvironment for dental tissue repair.

Cytokine priming

Stromal cell-derived factor-1 priming: Stromal cell-derived factor (SDF)-1, also known as chemokine CXC ligand (CXCL)12, plays a major role in cell trafficking and homing. SDF-1 has been shown to bind to the G-protein coupled receptor CXC receptor (CXCR)4 to induce SDF-1/CXCR4 signaling[103]. SDF-1 pretreatment enhanced proliferation, migration and differentiation, and inhibited apoptosis in DPSCs and PDLSCs [104-107]. Stimulating $CD105^+$ DPSCs with SDF-1 was shown to significantly improve the therapeutic effects in a canine pulpectomy model[108]. SDF-1-induced migration was reported to be mediated by the AKT and GSK3β/β-catenin pathways[109]. Autophagy is also involved in SDF-1-mediated DPSC migration during pulp regeneration[110]. In periodontal regeneration, Liang *et al*[111] demonstrated that cotreatment with SDF-1/exendin-4 facilitated the proliferation, migration, and osteogenic differentiation of PDLSCs and promoted periodontal bone regeneration. Similar effects on periodontal regeneration were also shown in studies of SDF-1 cotransplantion with bFGF[112] or parathyroid hormone (PTH)[113].

Granulocyte-colony stimulating factor priming: Granulocyte-colony stimulating factor (G-CSF) is a cytokine that stimulates the bone marrow to produce and release neutrophils into the bloodstream. G-CSF is frequently used to mobilize hematopoietic stem cells from the bone marrow to the systemic circulation[114]. G-CSF has a migratory effect on DPSCs similar to that of SDF-1, suggesting a potential alternative to SDF-1. Stimulation of MSC migration by G-CSF was demonstrated to strictly depend on the expression of G-CSF receptor (G-CSFR)[114]. It was revealed that 44%–56% of G-CSF-mobilized DPSCs were G-CSFR-positive cells[115]. G-CSF-mobilized DPSCs showed higher regenerative potential than untreated DPSCs[116].

Proinflammatory cytokine priming

IFN-γ priming: IFN-γ is a well-known proinflammatory cytokine secreted by activated T and NK cells. IFN-γ stimulates 2,3-indolamine dioxygenase (IDO) expression in MSCs to enhance immunosuppressive properties[117]. Wada *et al*[118] revealed that DPSCs, PDLSCs and gingival mesenchymal stem cells have immunosuppressive properties that are mediated partly by IFN-γ produced by activated peripheral blood mononuclear cells (PBMCs). Another study further demonstrated that the immunosuppressive effect of DPSCs on PBMC proliferation and B cell immunoglobulin production was significantly enhanced by IFN-γ and mediated by TGF-β [119]. Sonoda *et al*[120] found that DPSCs isolated from diseased teeth with pulpitis had impaired immunosuppressive abilities, but these abilities could be restored by IFN-γ treatment. This study revealed that IFN-γ improved dentin formation and T cell suppression of pulpitis-derived DPSCs by enhancing telomerase activity. In addition, other studies reported that healthy DPSCs exposed to IFN-γ exhibited increased proliferation and migration but impaired odonto/osteogenic differentiation, which may be regulated by the nuclear factor (NF)-κB and MAPK signaling pathways[121]. Increased release of CXCL6 and CXCL12 by IFN-γ-primed DPSCs may contribute to the homing of MSCs for pulp repair[122].

TNF-α priming: TNF-α is a pleiotropic cytokine produced predominantly by macrophages in response to bacterial endotoxin. TNF-α priming has a similar effect as that of IFN-γ priming and upregulates the expression of immunoregulatory factors, such as prostaglandin E2, IDO and hepatocyte growth factor (HGF)[123]. TNF signaling plays dual roles that are likely to be transduced through its two distinct receptors, TNFR1 and TNFR2. The interaction of TNF-α with TNFR1 mediates proinflammatory effects and cell death, while the interaction with TNFR2 mediates anti-inflammatory effects and cell survival. Recent studies have revealed that TNF–TNFR2 signaling but not TNF–TNFR1 signaling is a crucial mediator that regulates the regenerative and immunomodulatory effects of MSCs[124]. TNFR2 expression is corelated with NF-κB, which could be a possible explanation for the effect of TNF priming. The researchers confirmed the results by investigating the role of TNFR2 in proangiogenic functions, the suppression of T cells, the induction of Tregs, and alternations in T cell cytokine secretion pattern. Moreover, it was also mentioned by the authors that TNFR2 expression was essential after TNF pretreatment [125,126]. Paula-Silva *et al*[127] showed that 10 ng/mL TNF-α stimulated the differentiation of DPSCs toward an odontoblastic phenotype *via* the p38 signaling pathway while downregulating matrix metalloproteinase (MMP)-1 expression. The miR-21/STAT3 and NF-κB signaling pathways are reported to be involved during osteogenic differentiation[128,129]. Liu *et al*[130] revealed continuous transition in transcriptome changes during TNF-α mediated osteogenic differentiation. The TGF-β and PI3K/Akt pathways are sequentially activated. TNF-α (50 ng/mL) stimulated DPSC migration through upregulation of integrin α6[131]. However, the osteogenic differentiation of DPSCs was suppressed by high dose TNF-α (50–100 ng/mL) by activating Wnt/β-catenin signaling[132]. In addition to odontoblastic differentiation, TNF-α increased the angiogenic potential of cells in a coculture model of DPSCs and endothelial cells[133]. TNF-α in combination with lipopolysaccharide promoted angiogenesis *via* VEGF and sirtuin 1 signaling in DPSCs[134].

Growth factor priming

bFGF priming: bFGF, also known as FGF-2, is considered to be an important growth factor that assists tissue regeneration[135]. bFGF plays a potential role in the multipotent differentiation of DPSCs. Studies have reported that bFGF stimulates DPSC proliferation, angiogenesis, odontoblastic differentiation, and neuronal differentiation *in vitro* at concentrations of 20–50 ng/mL[136-138]. *In vivo* studies showed that bFGF at concentrations of 15 μg/mL–5 mg/mL significantly promoted angiogenesis [139,140] and odontoblastic differentiation[141,142] for pulp regeneration. Gorin *et al* [143] found that bFGF-treated DPSCs increased angiogenesis through HGF and VEGF secretion. bFGF priming augmented proangiogenic properties compared to the effect of hypoxia. Sagomonyants *et al*[138] reported that bFGF-induced mineralization of DPSCs was mediated by activation of the FGFR/MEK/ERK1/2 and BMP/BMPR signaling pathways. Zhang *et al*[144] revealed that bFGF induced neural differentiation in DPSCs by upregulation of nestin, MAP-2, β3-tubulin, glial intermediate filament protein (GFAP), and silent information regulator protein 1 expression through the ERK and AKT signaling pathways. bFGF assists DPSC differentiation by increasing the gene expression of MEPE, DSPP, DMP-1 and OCN[138]. bFGF is also involved in the production of hyaluronic acid (HA), which is a key component of the ECM. Tooth

development and odontoblastic differentiation require HA synthesis[145]. It is suggested that bFGF-primed DPSCs promote anti-inflammatory effects and odontoblastic differentiation through increased HA secretion[146,147]. Furthermore, bFGF-primed DPSCs exhibit altered expression of cytokines, including IL-6, IL-8, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, and CC chemokine ligand 20[148]. The PKC/PI3K–AKT/MAPK signaling pathways have been shown to contribute to cytokine upregulation[148]. Consistent with pulp regeneration studies, bFGF priming for periodontal tissue regeneration has shown similar results, including enhanced PDLSC migration, proliferation, osteogenesis and neurogenesis *in vitro* and *in vivo*[149-152].

However, some studies showed that bFGF did not benefit cell differentiation[142,153-155]. He *et al*[153] and Kim *et al*[142] showed that DPSCs preconditioned with 20 ng/mL bFGF exhibited increased cell proliferation, but inhibited cell differentiation. Likewise, Takeuchi *et al*[154] reported that bFGF promoted cell proliferation at a concentration of 50 ng/mL but inhibited cell differentiation at 100 ng/mL. Odontoblastic differentiation markers such as COLI[156], ALP activity[155], and calcium deposition were reported in some studies to have no changes[157]. Although there was a contradictory effect of bFGF on cell behaviors, most previous studies confirmed the potential effect of bFGF on cell proliferation. The effect of bFGF on cell differentiation was suggested to depend on the spatially and temporally controlled priming of MSCs by bFGF[158,159]. bFGF induced odontoblastic differentiation in dental pulp at the early stage (days 3–7)[138] but inhibited odontoblastic differentiation during late exposure (days 7–21)[158,159]. These findings suggested that the concentration of bFGF and the release duration should be accurately controlled.

Insulin-like growth factor-1 priming: Insulin-like growth factor (IGF)-1, a member of the insulin-like peptide family, has been shown to play an essential role in the growth and differentiation of various tissues, including teeth. Tooth germ explants treated with IGF-1 showed increased formation of dentin and enamel[160]. IGF-1 promoted DPSC proliferation and osteogenic differentiation by activating the mammalian target of rapamycin (mTOR) signaling pathway[161]. IGF-1 was also found to target EphrinB1 to regulate tertiary dentin formation[162]. Ma *et al*[163] further revealed the role of the IGF-1/IGF-1R/hsa-let-7c axis in regulating the committed differentiation of SCAPs. Yan *et al*[164] reported that IGF-1 rescued the adverse effects of high glucose concentration on DPSCs and protected against apoptosis. Regarding periodontal ligament regeneration, IGF-1 was shown to enhance the survival of PDLSCs[165]. Yu *et al*[166] described the beneficial effect of IGF-1 on PDLSC proliferation and osteogenesis *via* the ERK and JNK MAPK pathways.

Preconditioning with cytokines and growth factors is a promising way to improve the therapeutic efficacy of cells for dental tissue regeneration. However, these treatments synergistically or antagonistically influence MSC properties. Further and intensive studies need to be conducted to clarify the optimal concentration or combination of these factors and verify the detailed mechanisms according to their chemical characteristics.

Genetic modification of MSCs is an experimental technique that introduces exogenous DNA into MSCs to produce or overexpress specific factors[167]. This technique can enhance MSC survival and functions after transplantation, particularly in a hostile environment. To date, genes involved in survival, migration and regenerative properties have been mainly targeted in MSCs for dental tissue regeneration. Genetically modified dental MSCs were found to be more effective than wild-type cells. Here, we introduce several reports on enhancing the function and therapeutic effects of dental MSCs through gene modification.

Genetic modification to enhance retention and migration

It is generally accepted that transplanted MSCs are vulnerable to the harsh microenvironment *in vivo*; most cells can be cleared or become dysfunctional within a short time. This situation hinders the migration of transplanted MSCs to the target site to exert their effects. Therefore, enhancing cell retention and migration capabilities is crucial in improving the therapeutic efficacy of transplanted MSCs. The overexpression of genes related to apoptosis inhibition and self-renewal can be an effective method to achieve this goal. Factors secreted by genetically modified MSCs may exert therapeutic effects *via* paracrine actions.

One strategy to enhance the survivability of grafted DPSCs is to overexpress *Bcl-2*, an antiapoptotic gene that is important for maintaining cell viability. Several studies have demonstrated the effects of *Bcl-2* overexpression on proliferation, antiapoptosis, and osteo/odontogenic differentiation[168,169]. The forced expression of stemness-

rated genes such as *Sox2*, *Oct4* and *Nanog*, which contribute to the maintenance of pluripotency in embryonic stem cells, is reported to improve proliferation and prevent senescence in MSCs. Huang *el al*[170] showed that DPSCs overexpressing *Oct4* and *Nanog* exhibited enhanced proliferation, as well as osteogenic/chondrogenic/adipogenic differentiation. *Sox2*-overexpressing DPSCs showed beneficial effects on proliferation, migration and adhesion capability[171]. In general, MSCs transduced with pluripotent genes show remarkable benefits in their proliferation. However, conflicting results regarding differentiation potential and possible adverse effects such as tumor formation should be considered in the context of clinical applications[172, 173].

Forkhead box protein (Fox)O1 is a master regulator that mediates glucose metabolism, tumorigenesis, oxidative stress and bone formation[174]. Huang *et al*[175] investigated the role of FoxO1-transfected PDLSCs in regulating oxidative stress resistance and osteogenesis. The authors found that FoxO1 overexpression protected PDLSCs against oxidative damage and promoted ECM mineralization by increasing the expression of the osteogenic markers Runt-related transcription factor (Runx)2 and SP7 in an inflammatory environment. This study demonstrates the promising anti-inflammatory role of FoxO1 in periodontium regeneration for periodontitis treatment.

Genetic modification to modulate osteo/odontogenic differentiation

Lineage differentiation can be achieved *in vitro* by priming MSCs with extrinsic signaling molecules or by modifying culture conditions. Genetic modification of MSCs may be an alternative way to induce stable and effective lineage transdifferentiation [176]. BMP family members are regarded as crucial factors that initiate and maintain osteo/odontogenesis. Taşlı *et al*[177] carried out genetic modification of BMP2 and BMP7 in human tooth germ stem cells (hTGSCs). The researchers found that overexpression of BMP2 and BMP7 in hTGSCs led to enhanced expression of early markers of osteo/odontogenic differentiation, such as DSPP, OCN and COL1A. Yang *et al*[178-180] reported that DPSCs transfected with BMP2 showed increased expression of ALP, OCN, COL1A, BSP, DSPP and DMP1, indicating stimulation of osteo/odontogenic differentiation. *In vitro* tests showed that transfected DPSCs differentiated into odontoblast-like cells without osteogenic induction[180]. Zhang *et al*[181] investigated BMP2-transfected SCAPs. The modified SCAPs underwent cell differentiation toward the odontogenic lineage by upregulating the *ALP*, *OCN*, *DSPP* and *DMP1* genes. Another promising inducer of osteo/odontogenesis is growth/differentiation factor (GDF)11, also known as BMP11. Nakashima *et al*[182] reported that DPSCs overexpressing GDF11 exhibited induced expression of dentin sialoprotein and the formation of large amounts of reparative dentin in canine teeth. In addition, the authors demonstrated that a GDF11-transfected cell mass stimulated reparative dentin formation on the amputated pulp[183]. These results revealed the feasibility of using BMPs in gene-modified MSC applications for endodontic regeneration.

Runx2 is a crucial factor for bone formation and tooth development. Pan *et al*[184] demonstrated that Runx2-overexpressing DFCs upregulated osteoblast/cementoblast-rated genes and enhanced osteogenic differentiation *in vitro*. The authors also investigated the effects of mutant Runx2 without the VWRPY motif, which is responsible for suppressing transcriptional activation by Runx2. Overexpression of mutant Runx2 compared with full-length Runx2 led to higher expression levels of OPN, COLI and CP23 in DFSCs.

CONCLUSION

MSCs are increasingly being investigated as promising cell materials for tissue regeneration therapies due to their multilineage differentiation capabilities. MSCs derived from different dental tissues can be used as alternatives to bone marrow- and adipose tissue-derived MSCs. Numerous studies have demonstrated the high therapeutic potential of dental MSCs in various diseases, such as pulpitis, periapical, coronary artery, and neurodegenerative diseases.

Prior to clinical application, many efforts are still needed to focus on innovative strategies to maximize the therapeutic potential of MSCs. As summarized in this review, state-of-the-art technologies, including advanced culture systems, priming with cytokines and growth factors, and genetic editing, combined with understanding the therapeutic mechanism of dental MSCs, would significantly improve efficacy of MSC treatment in dental tissue regeneration. Overall, modified MSCs exhibit better therapeutic effects with high specificity on targets than ordinary cells. More studies

should be carried out to explore the therapeutic impact of the combined application of different modification or priming techniques to ensure improved outcomes and novel discoveries and further enhance therapeutic goals.

Researchers should also consider that MSC priming approaches must meet proper criteria and specific quality for clinical applications. The multilineage differentiation potential of MSCs is the required minimal criterion since it is a liability for many clinical applications. However, if MSCs are applied to treat neurodegenerative disorders, such as Parkinson's disease, then their osteogenic and adipogenic potentials are hazards. Thus, to utilize MSC products for specific applications, it is sometimes possible to lose certain MSC-defining characteristics through appropriate priming approaches[185]. Rather than focusing on the minimal criteria, as primed MSCs get closer to clinical application, they should meet the criteria that correlate with the safety and efficacy of the MSC product for treating specific diseases.

However, in preclinical trials, priming approaches for MSCs still have many limitations, including high costs, immunogenicity, donor-to-donor variability, variable source-dependent effects, and lack of good manufacturing practice (GMP) grade certification for clinical applications. Further studies are currently needed to evaluate the long-term *in vivo* tumorigenic potential of primed MSCs and the efficacy of each priming method for different clinical applications. Primed MSCs should meet qualified cell therapy standards and allow for GMP grade production while not compromise the quality attributes of the cells or be overly expensive. Ultimately, in conjunction with rigorous preclinical and clinical trials, primed MSCs can have enormous potential for wider applications in clinical settings in the future.

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REVIEW

Epigenetic regulation of dental pulp stem cells and its potential in regenerative endodontics

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Abstract

Regenerative endodontics (RE) therapy means physiologically replacing damaged pulp tissue and regaining functional dentin–pulp complex. Current clinical RE procedures recruit endogenous stem cells from the apical papilla, periodontal tissue, bone marrow and peripheral blood, with or without application of scaffolds and growth factors in the root canal space, resulting in cementum-like and bone-like tissue formation. Without the involvement of dental pulp stem cells (DPSCs), it is unlikely that functional pulp regeneration can be achieved, even though acceptable repair can be acquired. DPSCs, due to their specific odontogenic potential, high proliferation, neurovascular property, and easy accessibility, are considered as the most eligible cell source for dentin–pulp regeneration. The regenerative potential of DPSCs has been demonstrated by recent clinical progress. DPSC transplantation following pulpectomy has successfully reconstructed neurovascularized pulp that simulates the physiological structure of natural pulp. The self-renewal, proliferation, and odontogenic differentiation of DPSCs are under the control of a cascade of transcription factors. Over recent decades, epigenetic modulations implicating histone modifications, DNA methylation, and noncoding (nc)RNAs have manifested as a new layer of gene regulation. These modulations exhibit a profound effect on the cellular activities of DPSCs. In this review, we offer an overview about epigenetic regulation of the fate of DPSCs; in particular, on the proliferation, odontogenic differentiation, angiogenesis, and neurogenesis. We emphasize recent discoveries of epigenetic molecules that can alter DPSC status

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and promote pulp regeneration through manipulation over epigenetic profiles.

Key Words: Dental pulp stem cells; Regenerative endodontics; Epigenetic regulation; Noncoding RNAs; Histone deacetylase inhibitor; DNA methyltransferase inhibitor

Core Tip: We review the role of epigenetic modifications during fate determination of dental pulp stem cells, highlighting cellular processes implicating proliferation, odontogenesis, angiogenesis, and neurogenesis that are tightly correlated with regenerative endodontics (RE). We emphasize the potential of epigenetic manipulation through enzyme inhibitors in RE and provide insights for future development in regaining dental pulp function.

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INTRODUCTION

Regenerative endodontics (RE) has been defined as "biologically-based procedures designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp–dentin complex"[1]. The goal of RE is the restoration of the natural function of the dental pulp, including sensing exogenous stimuli, activating defense reactions, and forming reparative dentin, which contributes to the long-term preservation of natural teeth and dentition. Up till now, clinical RE procedures without cell transplantation, such as revascularization and cell homing, are capable of eliminating apical periodontitis. Yet most studies have indicated that they are unlikely to achieve pulp–dentin regeneration. Hence, there is an urgent desire to achieve pulp regeneration to develop a novel RE procedure that will not only solve apical periodontitis but also restore organized pulp–dentin complex structure and function. To this end, three essential elements of tissue engineering are recommended for further study: scaffold, growth factors, and stem cells. It has been demonstrated that pulp–dentin regeneration *in vivo* is achieved through RE with dental pulp stem cells (DPSCs). DPSCs were first isolated from dental pulp tissue of permanent third molars, namely permanent DPSCs (pDPSCs)[2]. Later, DPSCs were collected from human exfoliated deciduous teeth, which are named stem cells from human exfoliated deciduous teeth (SHED)[3]. Considering the remarkable potential of odontogenesis, vasculogenesis and neurogenesis *in vivo* and *in vitro*, DPSCs have been prized in pulp–dentin complex regeneration[4-6]. The latest clinical trial has detected pulp–dentin regeneration with blood vessels, sensory nerves, and lining odontoblast layer by implanting autologous SHED into necrotic immature permanent incisors. The regenerated dental pulp tissue promotes root elongation and apical foramen closure [7]. DPSCs have shown potential in pulp–dentin complex regeneration and have important directive significance for RE clinically.

It has been documented that odontogenesis is controlled by an intricate regulatory network composed of exogenous signaling stimuli, endogenous signaling molecules, and epigenetic regulators[8,9]. The epigenetic regulation, without DNA sequence changing, is made up of post-translational modifications of histones, DNA methylation, and nuclear regulatory ncRNAs[10]. Epigenetic regulation plays a crucial role in odontogenesis, eventually yielding the entire variety of dental tissues comprising complex teeth. Global epigenomes are indispensable to our understanding of gene regulation, cell fate determination, tooth development, and regeneration[11, 12]. The levels of acetylated histone H3 Lysine 9 (H3K9ac) and H3K27ac increase during odontoblast differentiation of mouse dental papilla cells. These changes are coordinated by the upregulation of histone acetyltransferase (HAT) p300 and downregulation of histone deacetylase (HDAC) 3[13]. The limited odontoblast differ-

entiation of DPSCs is enhanced by overexpression of *p300*[14] or knockdown of *HDACs*[15]. The application of HDAC inhibitor (HDACi) in promoting odontogenesis, such as odontoblast differentiation, has been highlighted in several studies. Entinostat (MS-275), a selective HDACi targeting HDAC1 and HDAC3, could induce DPSC odontoblast differentiation even without mineralization medium[16]. The significant mineralized tissue regenerative potential of HDACis has been confirmed in several animal models[17,18], and their application in RE is anticipated.

Therefore, a thorough understanding of this epigenetic regulation is important for researchers and endodontists to maximize the odontogenesis potential of DPSCs and fully realize pulp–dentin regeneration and RE. In this review, we discuss epigenetic mechanisms, including ncRNAs, histone modifications, and DNA methylation, and research progress in modulating cell fate determination of DPSCs. It has become clear that the regulation of epigenetic layer plays an essential role in the pulp–dentin regeneration based on DPSCs, and has potential in RE, which is also discussed.

PDPSCS, SHED AND THEIR CHARACTERISTICS

Dental tissue involves postnatal mesenchymal stem cells (MSCs) with easy accessibility and regenerative potential. Currently identified dental-derived stem cells include pDPSCs, SHED, periodontal ligament stem cells (PDLSCs), dental follicle progenitor stem cells (DFSCs), and stem cells from apical papilla[19]. They all possess osteogenic, adipogenic, and chondrogenic differentiation potential, along with a peculiar ability to form mineralized tissue. The translational clinical application of dental-derived stem cells in regenerative medicine has been broadly exploited. One direction is through bioscaffolds/biomaterials loaded with growth factors[20]. Another approach is to investigate the bio-induction effects of natural compounds such as polydatin, beer polyphenols on dental-derived stem cells[21,22].

The existence of stem cells in dental pulp was confirmed by pulp healing potential after injury and maintenance of tissue homeostasis. pDPSCs were initially identified in 2000[2]. Primary cultures of pDPSCs express endothelial (vascular cell adhesion molecule 1 and CD146), osteogenic [alkaline phosphatase (ALP), type I collagen, osteonectin, osteopontin (OPN), and osteocalcin (OCN)], and fibroblastic [type III collagen, and fibroblast growth factor (FGF)-2] markers. The bone matrix protein bone sialoprotein (BSP) and odontoblast-specific marker like dentin sialophosphoprotein (DSPP) are absent in pDPSC cultures, which confirms its undifferentiation status[2].

pDPSCs can be induced to form mineralized nodules when subjected to osteogenic medium[5]. Under adipogenic induction *in vitro*, pDPSCs form oil-red-O-positive lipid clusters and express high levels of nestin and glial fibrillary acid protein, suggesting that pDPSCs possess both adipogenic and neurogenic potential[23]. *Ex vivo* cultured pDPSCs acquire a neuronal morphology, and express neuron-specific markers under neuronal media conditions. When adding basic FGF (bFGF) and epidermal growth factor (EGF) to culture medium, pDPSCs transform to neural precursor cells that express the specific marker nestin[6]. When xenotransplanted into the chicken embryo and exposed to the endogenous neuronal microenvironment, pDPSCs show a neuronal morphology and migrate into facial structures and the central nervous system within the developing avian embryo[24]. When transplanted with hydroxyapatite/tricalcium phosphate (HA/TCP) powder into immunocompromised mice, the pDPSC transplants generate a dentin-like structure with highly organized collagenous matrix deposited around the odontoblast-like layer but do not indicate any hematopoiesis or initiate adipocyte formation. However, well-established vascularity is seen when transplanting tooth fragments containing pDPSC coated with synthetic scaffolds subcutaneously into nude mice[25,26]. When applying thermoresponsive hydrogels instead of polymer scaffolds, blood components are also produced in pulp-like tissues[27]. Another way to improve neovascularization for pulp regeneration is through fractionating $CD31^-/CD146^-$ cells from dental pulp; it turns out that $CD31^-/CD146^-$ canine DPSCs have greater angiogenesis potential when applied to the sectioned pulp of dogs[28]. A clinical experiment conducted on patients with irreversible pulpitis showed that pDPSCs transplantation in pulpectomized teeth induced positive response of electric pulp test and functional dentin formation as tested by cone beam computed tomography[29]. This study confirmed the safety and efficacy of DPSC-based RE.

Songtao Shi firstly documented the discovery and identification of SHED in 2003[3]. Histologically, SHED appear at the 6^{th} week of embryonic development, and consist of MSCs with multi-differentiating potential of adipocytes, chondrocytes, and

osteoblasts. SHED show even more extensive clonogenic, osteogenic potential than pDPSCs. Cotransplantation of SHED and HA/TCP material subcutaneously into immunocompromised mice produces similar dentin pulp-like tissues in accordance with pDPSCs. Other teams utilized human root canals to accommodate SHED that were coated with peptide hydrogel or collagen scaffold. They implanted this prepared root canal into immunocompromised mice, and new dentin was formed throughout the root canal and vascularization of pulp-like tissues was also identified[30]. SHED responded in a similar manner to pDPSCs during both *in vitro* neuronal induction and xenotransplantation into chicken embryos[6,24]. The breakthrough of DPSC-based pulp regeneration is associated with a clinical study in patients with pulp necrosis due to traumatic dental injuries. SHED implantation into injured incisor teeth regenerated dental pulp that recovered the formation of sensory nerves and blood vessels and ensured root elongation and closure of the apical foramen[7].

Besides the above multilineage differentiation potential, the expression of surface antigens provides another identification of DPSCs. DPSCs express MSC-specific markers such as STRO-1, CD90, CD44, CD73, CD90, CD105 and CD271[31]. These markers fail to distinguish DPSCs from other MSCs. Hematopoietic lineage markers such as CD34 and CD117, neurovascular markers such as glia 2 are also expressed in DPSCs[32]. Although these markers still lack specificity in distinguishing DPSCs, they provide further evidence for the regenerative potential of DPSCs.

DPSCs were collected from neonatal baby teeth (nDPSCs)[33] and dental bud (DBSCs)[5], and they exhibited more attractive stemness properties and higher proliferate rate, as indicated by more intensified expression of pluripotent markers such as v-myc avian myelocytomatosis viral oncogene homolog (c-Myc) and SRY-box transcription factor 2 (SOX2) compared to pDPSCs. When DBSCs underwent osteogenic induction, they expressed higher levels of c-Myc, SOX2, octamer-binding transcription factor 4, and homeobox transcription factor Nanog than pDPSCs expressed, suggesting that naive DPSCs hold functional advantages over pDPSCs[34].

Taken together, pDPSCs and SHED are the most widely studied DPSCs and have easier availability. They have neurovascular properties and unique odontoblastic and dentinogenic potential, which render them the most eligible stem cell source for pulp regeneration.

EPIGENETIC REGULATION OF DPSCS

The fate of DPSCs is strictly regulated on two levels: genetic control that involves signaling pathways and transcriptional factors; and epigenetic modulation that includes DNA methylation, histone modifications, and ncRNAs (Table 1 offers a summary of epigenetic enzymes in the fate determination of DPSCs). A thorough understanding of epigenetic modulation on DPSCs offers insights to manipulate DPSC fate towards pulp–dentin regeneration.

DNA methylation

DNA methylation refers to the covalent addition of a methyl group at the 5′ carbon of the cytosine by DNA methyltransferases (DNMTs, including DNMT1, DNMT3A, DNMT3B and DNMT3L). DNA methylation of promoters and enhancers leads to gene silencing by interfering with the binding of transcriptional factors or by chromatin structure remodeling[35]. The ten–eleven translocation (TET) family proteins (including TET1, TET2 and TET3) are responsible for the removal of the methyl group [36]. Gene expression is stringently controlled by the balance of methylation and demethylation.

DNA methylation states of pDPSCs, PDLSCs and DFSCs significantly differ, especially for surface antigens like CD109, and other factors implicating osteogenic pathways. PDLSCs express higher levels of osteogenic-related factors, a higher osteogenic potential *in vitro* and an enhanced mineralization capacity *in vivo*. Thus, the methylation profile is thought to be tightly correlated with differentiation potential [37]. When DNA methylation status is suppressed *via* pretreatment with 5-Aza-2′-deoxycytidine (5-Aza-CdR; a DNMT suppressor), pDPSCs exhibit receded proliferation and intensified mineralization and ALP activity under odontogenic induction [38]. Kruppel-like factor (KLF) 4 is an important regulator of cytodifferentiation and proliferation that promotes the odontoblastic differentiation and inhibits proliferation of pDPSCs[39]. The promoter region of *KLF4* is demethylated during odontoblastic differentiation, so as to facilitate the effective binding and transcriptional regulation of SP1[40]. The mRNA and protein expression level of TET1 increases during

Table 1 Enzymes related to epigenetic modifications of dental pulp stem cells activities

Proteins and their function	Epigenetic Targets	Gene manipulation of each epigenetic marker	Downstream targets	Biological process implicated	Reference
DNA methylation					
TET1, demethylation		Gene activation	*FAM20C*	(↑) proliferation, (↑) odontoblast differentiation	[41-43]
Histone methylation					
EHMT1, methylation	H3K9me2	Gene repression	*Runx2*	(↓) odontoblast differentiation	[51]
KMT2A, methylation	H3K4me3	Gene activation	*WNT5A*, *RUNX2*, *MSX2*, *DLX5*	(↑) odontoblast differentiation	[12,52]
EZH2, demethylation	H3K27me2/me3	Gene repression	Wnt/β-Catenin pathway, *IL-6*, *IL-8*, *CCL2*	(↑) Inflammation, (↓) odontoblast differentiation	[54-56]
KDM6B/JMJD3, demethylation	H3K27me3	Gene repression	*WNT5A*, *BMP2*	(↑) odontoblast differentiation	[52,58]
KDM5A, demethylation	H3K4me3/me2	Gene activation	*DMP1*, *DSPP*, *OSX*, *OCN*	(↓) odontoblast differentiation	[60]
Histone acetylation					
p300, acetylation	H3K9ac		*OCN*, *NANOG*, *SOX2*, *DSPP*, *Dmp1*, *Osx*	(↑) pluripotency, (↑) proliferation, (↑) odontoblast differentiation	[14,64]
HDAC3, deacetylation	H3K27ac		*Dmp1*, *Osx*	(↓) odontoblast differentiation	[13,66]
HDAC6, deacetylation				(↓) odontoblast differentiation	[15]

TET1: Ten-eleven translocation 1; EHMT1: Euchromatic histone lysine methyltransferase 1; KMT2A: Lysine methyltransferase 2A; KDM6B: Lysine demethylase 6B; KDM5A: Lysine demethylase 5A; p300: E1A binding protein p300; HDAC3: Histone deacetylase 3; HDAC6: Histone deacetylase 6; H3K9me2: Dimethylated histone H3 lysine 9; H3K4me3/me2: Tri-/di-methylated histone H3 lysine 4; H3K27me2/me3: Di-/tri-methylated histone H3 lysine 27; H3K9ac: Acetylated histone H3 lysine 9; H3K27ac: Acetylated histone H3 lysine 27; FAM20C: FAM20C golgi associated secretory pathway kinase; Runx2: RUNX family transcription factor 2; WNT5A: Wnt family member 5A; MSX2: Msh homeobox 2; DLX5: Distal-less homeobox 5; CCL2: C-C motif chemokine ligand 2; IL-6/-8: Interleukin 6/8; BMP2: Bone morphogenetic protein 2; DMP1: Dentin matrix acidic phosphoprotein 1; DSPP: Dentin sialophosphoprotein; OSX: Osterix; OCN: Osteocalcin; NANOG: Nanog homeobox; SOX2: SRY-box transcription factor 2.

odontogenic differentiation[41]. Knockdown of *TET1* inhibits pDPSC proliferation and impairs ALP activity, mineralized nodule formation, and decreases expression levels of DSPP and dentin matrix protein (DMP) 1 during odontogenic differentiation[42,43]. DNA methylation is also in charge of gene expression related to myogenic differentiation. Increased expression of myogenin, Myod1, and Pax7 is detected, along with myotube formation and myosin heavy chain expression after treating pDPSCs with 5-Aza-CdR. 5-Aza-CdR-mediated DNA demethylation induces skeletal myogenic differentiation of murine DPSCs *in vitro*[44]. Local injection of 5-Aza-CdR-pretreated pDPSCs into mice with cardiotoxin-induced muscle injury shows enhanced muscle regeneration[45].

Reparative dentin formation results from the delicate balance of inflammation and odontogenic differentiation. DNA methylation is involved in the inflammatory reaction of the human dental pulp as well. Administration of 5-Aza-CdR increases expression of inflammatory indicators interleukin (IL)-6 and IL-8 in lipopolysaccharide (LPS)-induced pDPSC inflammation. miRNA expression profile is altered by 5-Aza-CdR application. Among those differentially expressed miRNAs, miR-146a-5p is affected by DNA methylation[46]. In LPS-induced pDPSC inflammation, application of 5-Aza-CdR upregulates nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) signaling activity and stimulates inflammatory cytokine expression *via* demethylation of the promoter of an intracellular signal transducer, TNF-receptor-associated factor (TRAF) 6[47]. In lipoteichoic acid-treated pDPSCs, similar results are achieved with knockdown of *DNMT1* expression. Signal transducer MyD88 and

TRAF6 are both upregulated, but only the promoter of MyD88 is demethylated[48].

Histone modification

The tightly coiled DNA and histone cores (mainly H2A, H2B, H3 and H4) constitute a nucleosome, which functions as the fundamental subunit of chromatin. Different chemical modifications imparted on the histones result in alterations of chromatin architecture. To be specific, N termini of histone tails modified by methylation, acetylation, ubiquitination, phosphorylation, and other modifications of lysine and arginine residues can change the interaction among histones themselves or between histones and DNA[49].

Histone methylation: Histone methylation refers to the methylation of lysine or arginine residues of histone tails, which is regulated by histone methylases and demethylases[50]. It is the most widely studied histone modifications so far. pDPSCs and DFSCs respond differentially under mineralization induction. pDPSCs express higher levels of pluripotency-related genes and exhibit a faster rate of mineralization. Part of the explanation for this difference relies on different histone methylation profiles. Both cell types exhibit H3K4me3 (trimethylated histone H3 Lysine 4) active marks on early mineralization genes [runt related transcription factor (*RUNX*) 2, msh homeobox (*MSX*) 2, distal-less homeobox (*DLX*) 5], H3K9me3 or H3K27me3 on late mineralization markers [osterix (*OSX*), *BSP* and *OCN*], but H3K27me3 on odontogenic genes *DSPP* and *DMP1* are only seen in DFSCs[12]. CBFA2T2 (core-binding factor, runt domain, a subunit 2, translocated to 2) is upregulated during bone morphogenetic protein (BMP) 2-induced osteogenic differentiation of pDPSCs. CBFA2T2 is required for mineralization since it can inhibit euchromatic histone methyltransferase 1-mediated H3K9me2 on *RUNX2* promoter[51]. The bivalent histone domains of H3K4me3 and H3K27me3 on *WNT5A* promoter make the activation of *WNT5A* by the removal of H3K27me3 mark and increase of H3K4me3 mark on the promoter[52]. Ferutinin, a phytoestrogen extracted from *Ferula* species, has been used as an antibacterial, antioxidant, anti-inflammatory, and apoptosis-inducing agent. Pretreatment of ferutinin significantly increases H3K9ac and H3K4me3 in the promoter sites of the *WNT3A* and *DVL3* genes in pDPSCs and promotes osteogenic differentiation[53].

Enhancer of zeste homolog (EZH) 2 is specifically in charge of methylation of H3K27me3. EZH2 has been proved to participate in pulp tissue inflammation and regeneration[54]. Suppression of EZH2 function during TNF-α stimulation results in downregulation of proinflammatory factors and intensified osteogenic differentiation potential of pDPSCs[55]. EZH2-mediated H3K27me3 attenuates odontogenic differentiation of pDPSCs through modifying the β-catenin promoter and thus impairing the Wnt/β-catenin pathway[56]. The Jumonji domain-containing protein (JMJ) D3, also known as lysine-specific demethylase (KDM) 6B, removes the methyl marker of H3K27me2/3 specifically[57]. Overexpression of *JMJD3* promotes odontogenic commitment through combining with *BMP2* promoter site, removing H3K27me3 marker, leading to activation of genes associated with odontogenic differentiation[58]. When alcohol is added to mineralization-inducing media, the osteogenic potential of pDPSCs is inhibited *via* suppression of JMJD3[59]. H3K4me3 is another epigenetic mark related to odontogenic differentiation. Knockdown of *KDM5A*, an exclusive demethylase for H3K4me3, pDPSCs exhibited more intense ALP activity and more mineral deposition formation through the increment of H3K4me3 enrichment on odontogenic markers such as *DMP1*, *DSPP*, *OSX*, and *OCN*[60].

Histone acetylation: Histone acetylation is controlled by HATs and HDACs[61]. Eighteen human HDAC isoforms can be classified into three categories: class I (HDACs 1–3 and 8); class II (Zn-dependent enzymes, HDACs 4–7 and 9–11), and class III (sirtuins 1–7)[62]. Histone acetylation renders chromatin structure more favorable for transcriptional activation. Histone H3 acetylation is upregulated during odontogenic induction of pDPSCs[63]. The histone acetyltransferase p300 can activate *NANOG* and *SOX2* promoters and help maintain pDPSCs stemness. When pDPSCs are cultured in a normal medium, upregulation of p300 suppresses the expression of *DMP1*, *DSPP*, *DSP*, *OPN* and *OCN*. However, when they undergo odontoblastic differentiation, overexpression of *p300* leads to increased odontoblastic marker expression. p300 assembles at the promoter of *OCN* and *DSPP* and increases H3K9ac mark on *OCN* and *DSPP*[14]. Knockdown of *p300*, however, impairs ALP activity and mineralized nodule formation of pDPSCs during odontogenic differentiation[64]. Immediately after photo-biomodulation therapy on pDPSCs *in vitro*, H3K9ac is upregulated, which explains the improved viability and migration[65]. Another facet related to histone acetylation level is HDACs. When *HDAC6* is knocked down, the

ALP activity and mineralization potential of pDPSCs are increased[15]. When subjecting murine dental papilla mesenchymal cells to odontoblast induction, increased expression level of p300 and reduced HDAC3 expression are detected, leading to upregulated enrichment of H3K9ac and H3K27ac. HATs and HDACs modulate the process of dentinogenesis and odontogenic differentiation in a coordinated way[13]. Furthermore, p300 and HDAC3 modulate odontogenic differentiation in a time-specific manner through interacting with KLF4. At the differentiation initiation stage, HDAC3 acts on KLF4; thus DMP1 and OSX remain at a limited level. As the differentiation proceeds, HDAC3 translocates to the cytoplasm and KLF4 is able to bind with p300, transactivates *Dmp1* and *Osx*, ultimately enhances odontoblast differentiation[66].

ncRNAs

ncRNAs do not code for proteins, which can be categorized into small noncoding (snc) RNAs (< 200 nt) and long noncoding (lnc) RNAs (> 200 nt). sncRNAs can be further classified into miRNAs, PIWI-interacting (pi) RNAs, and siRNAs. miRNA and lncRNAs are two of the most-studied ncRNAs[67].

miRNAs: The processing of primary miRNA transcripts is initially tailored by two enzymes in the nucleus, known as Drosha and DGCR8, generating precursor (pre-) miRNAs. Subsequently, pre-miRNAs are exported to the cytoplasm and converted to mature miRNA duplexes by RNase III, namely Dicer. Mature miRNAs are combined into RNA-induced silencing complexes (RISCs). The incorporation of RISCs and the 3′ untranslated region (UTR) of specific mRNAs targets leads to gene repression by undermining mRNA stability or reducing translation[68,69].

Downregulation of miR-320b during calcium hydroxide stimulation can ease the inhibitory effect on the proliferation-related transcription factor Foxq1, leading to upregulation of Foxq1 and promoting the proliferation of pDPSCs[70]. miR-584 is another ncRNA that represses pDPSC growth, and it exerts this effect by targeting the 3′ UTR of PDZ-binding motif (TAZ)[71]. Sirtuin (*SIRT*) *7* is the downstream target of miR-152-mediated pDPSC senescence. Inhibition of miR-152 upregulates SIRT7 and represses pDPSC senescence[72]. Inhibition of miR-224 induces amplified MAPK8, caspase-3, caspase-9, and Fas ligand expression in pDPSCs, which is a sign of apoptosis, suggesting that miR-224 is essential for maintaining pDPSC viability[73]. Downregulation of miR-224 enhances pDPSC migration and proliferation[74].

The expression profiles of miRNAs in differentiated and undifferentiated DPSCs illustrate 22 differentially expressed miRNAs[75]. These miRNAs affect DPSC differentiation through various signaling pathways. Most identified miRNAs exert an inhibitory effect on odontogenic differentiation. Upregulation of miR-143 or miR-143-5p can attenuate osteogenic differentiation of pDPSCs, downstream inactivated pathways containing the NF-κB signaling pathway[76], osteoprotegerin receptor activator of the NF-κB ligand signaling pathway[77], and MAPK signaling pathway [78]. Disparate miRNAs can result in downregulation of the same signaling pathway, although their targets might be different. miR-488 and miRNA *let-7c* modulate the p38 MAPK signaling pathway; the former impacts MAPK1[79], and the latter downregulates insulin-like growth factor 1 receptor expression[80]. miR-215 and miR-219a-1-3p are both responsible for the cell-passage-related reduction of heat-shock protein B8 expression[81]. This reduction leads to weakened osteogenic differentiation capability of murine DPSCs[82]. Moreover, there are miRNAs that participate in the fate choice of pDPSCs in a multifaceted way. Among these miRNAs, miR-720 impacts the stemness of pDPSCs by inhibiting translation and stability of *NANOG* transcripts and repression of *DNMT3A* and *DNMT3B*. miR-720 mimics enhance osteogenic differentiation with intensified ALP activity, alizarin red staining, and increased expression of *ALP* and *OPN* and promotes proliferation of pDPSCs with an increased number of ki67-positive cells[83]. The modulation of miRNA in odontogenic differentiation is complicated, with multiple miRNAs, diverse signaling mechanisms, and disparate cellular processes. It provides both opportunities and challenges for precise miRNA-based regulation of dentinogenesis.

miRNAs participate in the regulation of angiogenesis and neovascularization under both physiological and pathological conditions[84]. When cultured in a medium supplemented with bFGF and vascular EGF (VEGF)-165, pDPSCs are induced toward endothelial differentiation, during which miR-424 is downregulated gradually, resulting in alleviation of the inhibitory effect on VEGF and kinase insert domain receptor expression[85]. 5-Aza-CdR can prompt myogenic differentiation of pDPSCs with a remarkable decrease of miR-135, and miR-143 expression. pDPSCs cotrans-

fected with miR-135 and miR-143 inhibitors acquire apparent myocyte properties even without administration of 5-Aza-CdR[86].

miRNAs are indispensable for the immunomodulation of dental pulp inflammation. The miRNA expression profile differs in healthy and inflamed dental pulp[87]. LPS or TNF-α *per se* can promote the odontoblastic differentiation of pDPSCs[88,89]. In most circumstances, the protective effects of miRNAs are realized through attenuating inflammatory reactions or promoting odontogenic differentiation. miR-223-3p is one of the markedly upregulated miRNAs in inflamed dental pulp as detected in clinically derived pulp tissues. Overexpression of miR-223-3p promotes odontoblastic differentiation of pDPSCs *in vitro*[90]. miR-506 and *let-7c-5p* confer a protective effect on LPS-induced inflammation of pDPSCs through decreasing expression of pro-inflammatory cytokines[91]. *In vivo* experiments have confirmed that *let-7c-5p* agomir decreases LPS-induced pulpitis in Sprague–Dawley rats[92]. Moreover, *let-7c-5p* possesses additional pro-osteogenesis potential in inflamed pDPSCs[93]. Knockdown of miR-140-5p increases odontoblastic differentiation and inhibits proliferation of pDPSCs under LPS stimulation. Toll-like receptor-4 is involved in the miR-140-5p-mediated effects on pDPSCs[94]. The expression of Fyn, a Src-family kinase associated with various types of inflammation, is upregulated in the microenvironment of deep caries. miR-125a-3p has been detected as the upstream factor of *Fyn* and identified as a positive factor regulating the odontoblastic differentiation of pDPSCs under TNF-α stimulation[95].

lncRNAs: lncRNAs can hardly be classified due to their diverse distribution in the genome and wide range of sizes. lncRNAs regulate gene expression at multiple levels, including transcriptional and post-transcriptional.

There are 139 differentially expressed genes between induced and undifferentiated human pDPSCs, with downstream pathways implicating cell cycle, extracellular matrix receptor interaction, and transforming growth factor (TGF)-β signaling pathways[96]. lncRNAs undergo transitional alterations during TNF-α-mediated osteogenic differentiation of pDPSCs, since lncRNA expression patterns differ after 7 and 14 d of treatment with TNF-α. These alterations in lncRNAs expression are predicted to be associated with mRNA alterations at day 7 and 14 posts TNF-α induction[97]. lncRNA DANCR declines with time during odontoblast-like differentiation of pDPSCs. The inhibitory effect of DANCR on odontogenic differentiation is realized through the inactivation of the Wnt/β-catenin signaling pathway. Downregulation of lncRNA DANCR has little impact on pDPSC proliferation but promotes the osteogenic, adipogenic and neurogenic differentiation of pDPSCs[98]. lncRNAs play a vital role in the angiogenesis of dental pulp and may be modulators of dental pulp angiogenesis. pDPSCs with normal culture and vascular induction show differential expression profiles of lncRNAs, which have been validated by microarray analysis [99]. Several proangiogenic factors including angiotensin, placental growth factor, FGF and EGF, are enriched in vascular differentiation, and they might serve as potential regulatory sites for lncRNAs.

It is worth noting that RNA methylation has emerged as an important post-translational modification mechanism on the fate determination of pDPSCs. Its discovery has brought a novel perspective of gene regulation. N6-methyladenosine (m^6A), is the most prevalent internal modification of mRNA. The addition and removal of methyl groups are mediated by methyltransferases and demethylases, and this structural alteration dynamically regulates various aspects of RNA metabolism, including changes in RNA folding, marking mRNA for decay, and facilitating the processing, maturation and translation of mRNA[100]. Downregulation of m^6A *via* depletion of methyltransferase 3 in pDPSCs significantly undermines the proliferation, migration and odontogenic differentiation of pDPSCs *in vitro*[101]. Upregulation of total m^6A content and methyltransferase 3 expression is observed in pDPSCs treated with LPS. When knocking down methyltransferase 3, LPS-induced NF-κB and MAPK signaling pathway activation is inhibited, along with decreased expression of proinflammatory cytokines[102], suggesting that RNA methylation is a promising target in the regulation of differentiation and immunomodulation of DPSCs.

EPIGENETIC REGULATORY NETWORKS IN THE FATE DETERMINATION OF DPSCS

Multiple direct and indirect connections exist between histone modifications, DNA methylation and ncRNAs. For instance, silencing of *MYT1* gene expression requires both EZH2 and DNMTs. EZH2 assists the binding of DNA methyltransferases and

facilitates CpG methylation of EZH2-target promoters[103]. Specific protein–RNA interactions with lncRNA are responsible for the initiation of deposition of polycomb-repression-complex-2-mediated H3K27me3[104].

During the fate determination of pDPSCs, complicated epigenetic networks regarding lncRNAs, miRNAs and DNA methylation have been revealed in recent studies. lncRNA G043225 promotes odontogenic differentiation of pDPSCs *via* directly binding to miR-588 and fibrillin 1[105]. lncRNA H19 can repress the activity of DNMT3B, reduce the methylation level of *DLX3*, and thus lead to the promotion of odontogenic differentiation of pDPSCs[106]. Similar mechanisms can be applied to miR-675, which is capable of inhibiting DNMT3B-mediated methylation of *DLX3* to promote odontogenic differentiation of human DPSCs[107]. lncRNA CCAT1 is upregulated in pDPSCs and promotes cell proliferation and differentiation by repressing the expression of miR-218, an antiodontogenic factor[108].

THERAPEUTIC APPLICATION OF EPIGENETIC MODIFICATION IN RE

RE is a biological process that aims to regain both structure and function of the dentin–pulp complex. There have been extensive searches for novel bio-inductive approaches for the regeneration of damaged dental tissues over recent years. The process of RE requires a microenvironment conducive to repair, agents with anti-inflammatory properties, induction of mineralization, angiogenesis and neurogenesis, and recruitment and differentiation of DPSCs. The discovery of novel factors, which manipulate epigenetic modulation and contribute to inducing DPSCs toward odontogenic differentiation, angiogenesis and neurogenesis would accelerate research in RE.

Kuang *et al*[26] performed RE on first molars of rats by implantation of hypoxia-primed pDPSCs blended with a synthetic polymer and found pulp-like tissues histologically, and vascularization were generated in this *in situ* model. Another team performed autogenous transplantation of the BMP2-treated DPSCs culture onto the amputated canine pulp of dogs. The BMP2 pretreated group produced odontoblast-like cells with long processes attached to the osteodentin and formed tubular dentin [109]. There are subsets of progenitor cells derived from dental pulp that exhibited greater angiogenic and neurogenic potential; for instance, $CD105^+$ DPSCs. $CD105^+$ DPSCs were fractionated by flow cytometry and further transplanted in canine teeth after pulpectomy with the addition of stromal cell-derived factor-1 and collagen mixtures. Regenerated pulp including nerves and vasculature was produced, followed by new dentin formation along the dentinal wall[110]. The granulocyte-colony stimulating factor (G-CSF) is capable of sorting out $CD105^+$ DPSCs. Ectopic tooth root transplantation of DPSCs subset mobilized by G-CSF in immunodeficient mice exhibited larger fibrous matrix formation and larger neovascularization compared with unsorted DPSCs[111]. These results suggest that preconditioned pDPSCs during the process of RE might guide differentiation specifically and ensure optimal functional pulp regeneration. Despite the inspiring and cheerful outcomes of clinical experiments carried out by Xuan *et al*[7], long-term follow-up of autologous SHED-based RE is required. Besides, self-derived DPSCs sources are limited. The efficacy and safety of allogenic DPSC transplantation need to be explored. Preconditioning of DPSCs with epigenetic molecules to optimize pulp regeneration might offer solutions to those problems[112].

Histone acetylation and HDACis in RE

HDACis have received intensive focus as potential agents for the treatment of cancer [113], inflammatory disease[114], and neurodegenerative disorders[115]. HDACs play a crucial part in the modulation of dental pulp development and repair. HDACis, as small molecules, have been put forward as an agent for pulp–dentin regeneration (Table 2). There are basically two types of HDACis: pan-HDACis and isoform-specific HDACis. Valproic acid (VPA), suberoylanilide hydroxamic acid (vorinostat, SAHA) and trichostatin A (TSA) are pan-HDACis that have been extensively studied to promote mineralization and differentiation at low concentrations[116,117]. Exposure of pDPSCs to 1 mmol/L VPA or 20 nmol/L TSA promotes cell proliferation, migration and adhesion[118]. VPA (0.125–5 mmol/L) and TSA (12.5–400 nmol/L) significantly increases mineralization in a dose-dependent manner[116,119]. Low concentration of VPA promotes matrix mineralization through selective inhibition of HDAC2 over HDAC1. To mimic 3D tissue formation, Paino *et al*[117] exploited Gingistat collagen sponges to grow pDPSCs. The seeded scaffolds were bathed in osteogenic medium

Table 2 Epigenetic molecules promoting odontogenic differentiation of dental pulp stem cells

	Targets	Cell population	Medium	Upregulated odontogenic genes	Downregulated odontogenic genes	Reference
HDACis						
TSA	HDAC 1, 2, 3 (Class I); HDAC 4, 5, 6 (Class II)	pDPSCs	MM	*DMP1*, *DSPP*, *BSP*	*OCN*	[124]
		Murine MDPC23	MM	*Dmp1*		[119]
		Rodent primary dental pulp cells	MM	*Bmp4*, *Dspp*, *Bmp2*, *Opn*		[116]
		Rodent MDPC23	GM	*Bmp4*, *Ocn*, *Dmp1*, *Runx2*		[16]
SAHA	classes I and II	Murine MDPC23	MM	*Nfic*, *Dspp*, *Alp*, *Dmp1*, *nestin*		[122]
VPA	HDAC 1, 2, 3 (class I)	pDPSCs	GM, MM	*BSP*, *OPN*	*OCN*	[117]
		Murine MDPC23	MM	*Dmp1*, *Bmp4*, *Tgfβ1*		[119]
		rodent MDPC23	GM	*Bmp2/4*, *Ocn*, *Runx2*		[16]
		Rodent primary DPSCs	MM	*Dmp1*, *Bmp2*, *Bmp4*, *Dspp* , *Opn*		[116]
MS-275	HDAC 1, 3 (class I)	pDPSCs	GM	*RUNX2*, *DMP1*, *ALP*, *DSPP*		[126]
		rodent MDPC23	GM	*Bmp2/4*, *Col1α1*, *Ocn*, *Dmp1*, *Dspp*, *Runx2*, *Klf5*, *Msx1*		[16]
LMK-235	HDAC 4, 5 (class II)	pDPSCs	GM	*ALP*, *DSPP*		[127]
			MM	*ALP*, *DSPP*, *RUNX2*		[127]
DNMTis						
5-Aza-CdR		pDPSCs	MM	*DSPP*, *DMP1*, *OSX*, *DLX5*, *RUNX2*		[38]
RG-108		Murine mDPC6T	MM	*Klf4*, *Dspp*, *Dmp1*		[40]

HDACis: Histone deacetylase inhibitors; TSA: Trichostatin A; SAHA: Suberoylanilide hydroxamic acid; VPA: Valproic acid; MS-275: Entinostat; DNMTis: DNA methyltransferases inhibitors; 5-Aza-CdR: 5-Aza-2′-deoxycytidine; HDAC: Histone deacetylase; pDPSCs: Dental pulp stem cells from permanent teeth; MDPC23: Murine odontoblast-like cell line; mDPC6T: Murine preodontoblast cell line; MM: Mineralized medium; GM: Growth medium; DMP1: Dentin matrix acidic phosphoprotein 1; DSPP: Dentin sialophosphoprotein; BSP: Bone sialoprotein; BMP4: Bone morphogenetic protein 4; BMP2: Bone morphogenetic protein 2; OPN: Osteopontin; Runx2: RUNX Family transcription factor 2; Nfic: Nuclear factor I C; ALP: Alkaline phosphatase; Tgfβ1: Transforming growth factor beta 1; OCN: Osteocalcin; Col1α1: Collagen type I alpha 1; Klf5: Kruppel like factor 5; MSX1: Msh homeobox 1.

supplemented with VPA for 30 d. More intense calcium deposits were observed in this system. pDPSCs preconditioned with HDACi (VPA, TSA or SAHA) and 15-d osteogenic induction were transplanted subcutaneously into immunodeficient mice. VPA treatment produced a well-organized lamellar bone tissue although a decrease of OCN expression was observed[120]. SAHA, an FDA-approved drug for treatment of lymphoma, mainly acts on class I and II HDACs[121]. Addition of SAHA to culture medium enhances matrix mineralization and expression levels of odontoblast marker genes during odontoblast differentiation of MDPC23 cells, which is an odontoblast-like cell line[122]. Similar results were found during mineralization induction of murine DPSCs. Moreover, short-term SAHA treatment promotes mineralization without loss of cell viability, while long-term SAHA inhibits differentiation. Low dose (1 μmol/L) SAHA even promotes cell migration[123]. TSA enhances pDPSCs proliferation *via* activation of the JNK/c-Jun pathway and promotes DPSC differentiation and increased expression of *DSPP*, *DMP1*, *BSP* and *OCN in vitro* through affecting Smad2/3- and NFI-C-related signaling pathways. TSA can promote odontoblast differentiation and dentin formation *in vivo*. Neonatal mice with maternal exposure to TSA exhibited thicker dentin, larger dentin areas, and higher odontoblast numbers in their postnatal molars with stronger DSP expression[124]. Apart from regulation of DPSC

gene expression, VPA, SAHA and TSA can promote pulp–dentin repair through facilitating the release of dentinal matrix components from dentin. Although they are not as effective as EDTA treatment, each of them shows different extraction profiles [125].

MS-275 is a selective HDACi that targets HDAC1 and HDAC3. Administration of MS-275 to pDPSCs under normal culture can induce upregulation of odontogenesis-associated proteins expression, including RUNX2, DMP1, ALP, and DSPP. Cytotoxicity can be avoided at a concentration of 20 nmol/L. The MAPK signaling system was barely activated under MS-275 stimulation, suggesting that MS-275 induces odontogenesis independent of MAPK signaling[126]. The pro-odontogenic potential of MS-275 was also tested on a murine odontoblast-like cell line, MDPC-23. Without the induction of mineralization medium, MS-275 alone was capable of increasing expression of *Bmp2*, *Bmp4*, *Col1α1*, *Ocn*, *Dmp1*, *Dspp*, *Runx2*, *Klf5*, and *Msx1*, with elevated ALP activity and intensified calcified nodule formation[16]. Isoform-specific agents like LMK-235 selectively inhibit HDAC4 and HDAC5. LMK-235 at 100 nmol/L barely affected the proliferation of pDPSCs, but possessed pro-odontogenic potential. Odontoblast markers (ALP, DSPP, and RUNX2) were downregulated when the concentration increased. Expression of OCN was not affected by LMK-235 administration, indicating that LMK-235 might act on early stages of odontogenic differentiation. LMK-235 combined with mineralization induction medium enhances odontoblastic marker expression of pDPSCs[127]. As topical agents for pulp repair, HDACis generally exhibit low toxicity since non-cancer cells are resistant to HDACi-mediated apoptosis compared to cancerous cells[128]. Adverse effects such as fatigue, nausea, and hypocalcemia due to high-dose systemic administration of HDACi can also be avoided.

The presence of either 5-Aza-CdR or TSA can increase expression of the endothelial marker genes in bone-marrow-derived multipotent adult progenitor cells in basal differentiation medium, which indicates the possibility of HDACi-induced angiogenesis[129]. VPA treatment can enhance sciatic nerve regeneration and recovery of motor function in adult rats[130]. VPA tends to induce neuronal differentiation and inhibit glial differentiation of adult hippocampal neural progenitors *via* acetylation of histone H4 associated with proneural genes[131]. HDACis have emerged as potent contenders in the treatment of chronic immune and inflammatory disorders, including rheumatoid arthritis, psoriasis, inflammatory bowel disease, and multiple sclerosis [132]. The underlying mechanism is still controversial but possibly relies on reduced inflammatory cytokines and nitric oxide production and inhibition of NF-κB transcriptional activity[133]. The angiogenesis, neurogenesis, and immunomodulatory potential of HDACis on inflamed dental pulp remain to be explored, but these characteristics cater to the requirements of pulp regeneration.

The mineralized tissue regenerative potential of HDACis has been tested on several animal models. Huynh *et al*[134] conducted an experiment on a murine model with calvarial defects. They seeded human periodontal ligament cells, which were preincubated with TSA in growth medium, onto a scaffold to induce repair. Apparent bone formation was detected 4 wk after implantation[134]. Topical application of MS-275 to calvarial defects of Sprague–Dawley rats stimulated bone formation. Collagen sponges loaded with MS-275 were applied to the injury site, in which significant bone healing was observed in the MS-275-treated groups in a dose-dependent manner. The pro-osteogenic capacity of MS-275 was demonstrated in an osteoporosis mouse model induced by soluble receptor activator of NF-κB ligand, as consecutive injection of MS-275 recovered bone volume, thickness, and separation[17] . Promising outcomes of MS-275 in bone regeneration promote the analysis of its potential in RE.

DNA methylation and DNMT inhibitors in RE

Inhibition of DNMTs has been demonstrated to enhance odontoblast differentiation. There are two DNMT inhibitors that affect the differentiation potential of pDPSCs, 5-Aza-CdR and RG108 (Table 2). They were initially identified as antitumor agents and were used for the treatment of leukemia and myelodysplastic syndromes[135]. When administered in combination with odontogenic medium, 5-Aza-CdR intensifies the expression of odontogenic markers and promotes ALP activity and mineral nodule formation[38]. The pro-odontogenic effect of RG108 was tested in a murine preodontoblast cell line, mDPC6T. RG108 is effective in suppressing DNMT activity and promotes odontogenic differentiation[40]. Melatonin, N-acetyl-5-methoxytryptamine, an endogenous hormone mainly in charge of circadian rhythms, decreases DNMT expression. Melatonin can cause global DNA hypomethylation and promote odontogenic differentiation of pDPSCs *in vitro*[136]. Although DNMT inhibitors show angiostatic activity when utilized as antitumor agents[137], they may show an

opposite effect on stem cells. 5-Aza-CdR downregulates protein expression of the pluripotency gene *Oct4* and upregulates protein expression of endothelial cell marker genes in differentiated mouse embryonic stem cells (ESCs). With the involvement of 5-Aza-CdR, differentiated ESCs form capillary-like structures when plated on Matrigel [138]. When treating human bone-marrow-derived MSCs with 5-Aza-CdR, reactivation of endothelial cells specification occurs and arterial marker gene expression level is elevated, accompanied by tube-like structure formation[139]. These small molecules have been indicated applicable in pulp tissue regeneration, but animal studies are necessary to determine whether DNMT inhibitors can be utilized as regenerative agents.

ncRNA-based RE

Numerous miRNAs and lncRNAs are actively involved in dentin formation and pulp mineralization processes during tooth development and pulp repair, and are crucial for inflammation control and immunomodulation. Emerging evidence has shown that ncRNAs are critical for angiogenesis and neovascularization, both in health and disease contexts[84]. miRNAs have the ability to regulate the migration, proliferation, and differentiation of endothelial cells[140,141]. lncRNAs such as MANTIS and GATA6-AS can promote angiogenic sprouting[142,143]. Consequently, miRNAs and lncRNAs have therapeutic potential in RE. However, their susceptibility to nucleases and poor penetration into cell membranes largely restrict their clinical application [144]. Effective delivery of therapeutic miRNAs has aroused much interest over recent decades. With the development of biotechnology and pharmaceutical progress, substantial approaches are being invented to deliver miRNAs: virus-based delivery, nonviral delivery (lipid nanocarriers, biomaterials, or chemical modifications), and exosome-based delivery systems[144,145]. It can be expected that an increasing number of ncRNA-targeting therapeutics will progress through clinical development in the upcoming years.

When it comes to ncRNA-targeting therapeutics in RE, ncRNA delivery systems highlight topical instead of the systemic application. This is a nascent topic. A team has developed a serum-endurable magnetic GCC-Fe_3O_4 nanocarrier and tested it on cultured pDPSCs, and found that this carrier has delivered miR-218 mimics/inhibitors into cells efficiently with low cytotoxicity[146]. Although further *in vivo* experiments are needed to confirm its efficacy, this could be a promising start. More effort needs to be made in ncRNA-targeting RE until it is clinically feasible.

CHALLENGES OF EPIGENETIC APPROACHES IN PULP REGENERATION

Although epigenetic agents are capable of modulating multiple biological processes implicating proliferation, multi-lineage differentiation, migration, and immunoregulation, which is promising in the process of tissue regeneration, several regulatory obstacles and translational challenges need to be resolved before clinical application. Firstly, the off-target effects cannot be ignored. The majority of HDACis like VPA, SAHA and TSA are pan-inhibitors without specific selectivity, which leads to general upregulation of histone acetylation. Single miRNAs can silence multiple target genes; for instance, during the process of immunomodulation, one miRNA can overexpress anti-inflammatory factors and at the same time upregulate proinflammatory cytokines [147]. Secondly, there are concerns about neoplastic transformation during the process of regeneration. The targets of epigenetic-modulating agents need to be screened and investigated thoroughly so as to minimize unwanted effects before the clinical application of epigenetic therapeutics. Lastly, it requires both financial and technical support to bank DPSCs and reserve this "biological insurance". Standardized and optimized manufacturing protocols need to be established to manage the procedures of collection, isolation, expansion, and cryopreservation and to ensure the quality of cell sources[148].

CONCLUSION

The process of dentin-pulp regeneration relies on stem cells with proliferation and pluripotency capacity, signaling molecules that can regulate cellular fate and scaffold which offers a favorable microenvironment. Complicated regulatory networks of histone modifications, DNA methylation, and ncRNAs are involved in guiding dentin-

pulp regeneration. A thorough understanding of epigenetic regulation in the orchestration of DPSC fate will facilitate the self-renewal, migration, and multi-differentiation of DPSCs during pulp tissue regeneration. Cheerful results of HDACis, such as TSA and MS-275, on bone repair have been achieved in animal models, and thus *in vivo* pulp-dentin regeneration of HDACis can be anticipated. The regenerative potential of DNMTis and ncRNAs is still absent *in vivo* studies. However, risks concerning the delivery system, off-targets, and neoplastic transformation are vigorous research fields and need to be tackled before epigenetic strategies applied in optimizing dentin-pulp regeneration. The epigenetic manipulation of DPSCs towards differentiation and regeneration with small molecules will be a hopeful direction in the search for approaches of functional pulp reconstruction.

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REVIEW

Effects of immune cells on mesenchymal stem cells during fracture healing

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Abstract

In vertebrates, bone is considered an osteoimmune system which encompasses functions of a locomotive organ, a mineral reservoir, a hormonal organ, a stem cell pool and a cradle for immune cells. This osteoimmune system is based on cooperatively acting bone and immune cells, cohabitating within the bone marrow. They are highly interdependent, a fact that is confounded by shared progenitors, mediators, and signaling pathways. Successful fracture healing requires the participation of all the precursors, immune and bone cells found in the osteoimmune system. Recent evidence demonstrated that changes of the immune cell composition and function may negatively influence bone healing. In this review, first the interplay between different immune cell types and osteoprogenitor cells will be elaborated more closely. The separate paragraphs focus on the specific cell types, starting with the cells of the innate immune response followed by cells of the adaptive immune response, and the complement system as mediator between

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them. Finally, a brief overview on the challenges of preclinical testing of immune-based therapeutic strategies to support fracture healing will be given.

Key Words: Trauma; Bones; Immune response; Mesenchymal stem cells; Fracture healing

Core Tip: There is substantial evidence that immune cells influence mesenchymal stem cells (MSCs) after trauma. Bone is considered as an osteoimmune system based on cooperatively acting bone and immune cells, cohabitating within the bone marrow. The subdivisions are highly interdependent, sharing progenitors, mediators, and signaling pathways. During fracture healing many different cell types categorized to the innate and adaptive immune system interact. MSCs with their manifold immunomodulatory and regenerative properties serve as progenitors for fibroblasts, chondrocytes and osteoblasts. The alterations in the immune response usually become apparent early in the healing process of a fracture. This opens new avenues for early interventions.

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INTRODUCTION

In vertebrates, bone can be considered as an osteoimmune system encompassing functions of a locomotive organ, a mineral reservoir, a hormonal organ, a stem cell reservoir and a cradle for immune cells. This osteoimmune system is based on cooperatively acting bone and immune cells, cohabitating within the bone marrow. They are highly interdependent, a fact that is confounded by shared progenitors and signaling pathways. Receptor activator of nuclear factor kappa B ligand (RANKL) is a well described example of this interdependency. Well known as a key factor for osteoclast differentiation, RANKL also regulates T-cell differentiation and proliferation[1]. Mesenchymal stem cells (MSC) are adult stem cells. In bone, MSCs are traditionally found in the bone marrow. They act as precursor cells for chondrogenic, adipogenic and osteogenic cells during bone homeostasis and fracture healing[2]. However, MSCs also play crucial roles in hematopoiesis and maintenance of immune cell progenitors [3]. Recently, different MSC subsets with distinct roles on different immune cell progenitors have been described[4,5]. In MSCs, this process seems to be strongly dependent on the expression of stem cell growth factor, also known as C-type lectin domain family 11 member A or osteolectin. MSCs lacking this factor seem to regulate hematopoiesis, while MSCs expressing osteolectin strongly influence maintenance and function of common lymphoid progenitor cells and their progenies[4]. Furthermore, these osteolectin positive MSCs, prone to undergoing osteogenesis[5], increase in number following a bone fracture and thus, represent the main source of osteoprogenitor cells during fracture healing[4].

The bone-immune cell interplay at first considered the role of the innate immune system but more and more the role of the adaptive immune system was investigated. With respect to the adaptive immune system, the question arises whether it has beneficial or detrimental effects on regeneration. Considering evolution, the development of the immune system coincides with a decline in the regenerative capacity — *i.e.* the more elaborate the immune response the less capable of regeneration an organism is[6]. A hallmark of adaptive immunity is its capacity to "remember" pathogens. This immune memory is only present in vertebrates, which constitute 1%-2% of the living species[7]. Indeed, while their immune system is very elaborate, vertebrates only have a very limited regenerative capacity. An exception are mammalian embryos, which, still lacking adaptive immune responses, are capable of scar-free regeneration, a capacity that diminishes after birth and with aging[8]. This indicates that adaptive immunity is rather unfavorable for regeneration.

In human adults, most tissues heal with scar tissue formation. Bone, however, is capable of healing without scar tissue formation. Bone healing is a highly complex process consisting of numerous well-orchestrated interdepending and overlapping steps, which, if undisturbed, result in tissue fully restored in form and function[9]. Using endochondral bone regeneration as a model, effects of the innate and adaptive immune system have been investigated to understand their role in regenerative processes and to explore the possibility of using immune-modulatory strategies for the development of new therapeutic approaches. Upon bone injury and inadvertent vessel rupture, the initial step in healing consists of coagulation and the coupled release of platelet-derived pro-inflammatory cytokines, *e.g.*, interleukin (IL)-6, or tumor necrosis factor (TNF)-α[10]. The main functions of the clotting reaction are the closure of a possible breach of the outer hull of an individual and a strong defense against possible invading pathogens. The released cytokines stimulate homing of leukocytes into the fracture site (Figure 1). With vessel rupture, the supply of oxygen and nutrients is diminished at the injury site. Thus, cells active in this initial phase of bone healing need to be capable of functioning in this detrimental environment (low pH, low oxygen, disturbed sodium and potassium balance[11,12]).

Neutrophils, the most abundant immune cells, are the first cells to arrive at inflammatory sites. The fracture hematoma was shown to be a strong inducer of neutrophil homing[13,14]. Within less than an hour, they are recruited to the site of fracture[15], triggered by damage associated molecular patterns (DAMPs), including mitochondrial DNA (deoxyribonucleic acid) fragments[16]. Equipped with highly potent activities such as phagocytosis, respiratory burst, or neutrophil extracellular trap (NET) formation, neutrophils strongly contribute to the first inflammatory reaction and formation of granulation tissue. Additional recruitment of fresh neutrophils from bone marrow by the C-X-C chemokine receptor (CXCR) type 4 (CXCR4)-C-X-C motif chemokine ligand (CXCL) 12 (CXCL12) axis, further supports the inflammatory reaction[17]. This inflammatory reaction at the beginning of the healing process has a substantial influence on the whole process[18]. As a consequence of the systemic inflammatory reaction, neutrophils invade not only the site of injury but also lung and liver tissue rapidly after a fracture[19]. Almost simultaneous to neutrophils, mast cells (MCs) and dendritic cells (DCs) appear in the fracture hematoma. As tissue-resident hematopoietic cells, MCs modulate innate and adaptive immune responses, not only in the early inflammatory phase of fracture healing, but also later during bone remodeling, where MCs exert several functions in the regulation of angiogenesis, osteogenesis, and osteoclastogenesis[20]. Shortly after, natural killer (NK) cells arrive at the site of injury, attracted by pro-inflammatory cytokines TNF-α and IL-6[21]. The role of NK cells in fracture healing is not fully understood and seems strongly dependent on the current inflammatory status. Within one day, monocytes are recruited to the fracture hematoma. They first differentiate into pro-inflammatory M1 macrophages, contributing to the clearance of the granulation tissue by phagocytosis. During callus formation, anti-inflammatory M2 macrophages are present, supporting migration of osteoprogenitor cells, matrix formation, and angiogenesis[22]. As precursors for osteoclasts, monocytes/macrophages also contribute to the final remodeling phase of fracture healing. Osteoclast differentiation is strongly dependent on factors secreted by B- and T-lymphocytes, which invade the fracture site, when granulation tissue is fully accomplished. In their pro-inflammatory state, T cells may act in the fracture environment[23,24].

This initial pro-inflammatory reaction is required to initiate fracture healing, not only for defense purposes but also to attract cells needed in the downstream healing process. However, this process needs to be tightly monitored, because the pro-inflammatory reaction becomes detrimental if it is too strong or lasts too long[25]. Under anaerobic conditions the transcription factor, hypoxia inducible factor 1α, is stabilized and induces the expression of angiogenic factors by cells in the fracture site. This initiates the essential revascularization step by homing of endothelial progenitor cells (EPCs). Expression analysis revealed that upregulation of angiogenic factors is paralleled by enhanced anti-inflammatory signaling, to terminate the initial pro-inflammatory reaction in order to proceed towards the next healing phase[9]. A switch from pro- to anti-inflammatory signaling is achieved, *i.e.*, by an upregulation of IL-4 and IL-13, which support the Th2 (T helper cells type 2) and M2 phenotype in T cells and macrophages[26]. Influencing the initial immune reaction by local application of these cytokines in a mouse osteotomy model significantly improved healing[22], emphasizing the importance of a tightly regulated initial inflammatory reaction and of swift downregulation of the initial pro-inflammatory reaction for a successful healing outcome. Thus, repeated irritation of the fracture hematoma can favor non-union[27]. Furthermore, movement of the early fracture hematoma into muscle tissue can induce

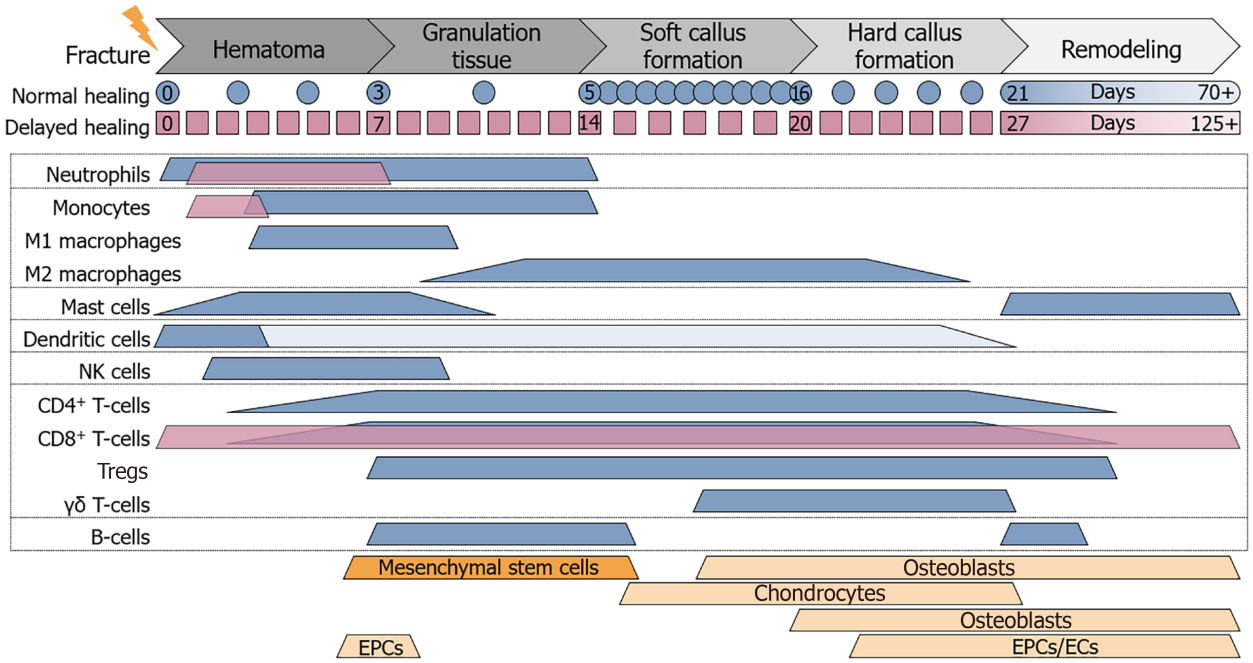

Figure 1 Cell composition at the site of fracture. During the different phases of fracture healing the cell composition at the site of fracture changes. Expected timeline of normal (blue) and delayed (magenta) fracture healing is depicted below the phases of fracture healing. Colored (blue and magenta) beams representing the timeframe where immune cells are expected to be active at the site of fracture, based on different *in vivo* studies. Orange beams representing the timeframe where mesenchymal stem cells, chondrocytes, osteoblasts, osteoclasts, endothelial progenitor cells, and endothelial cells are involved in the fracture healing process. CD: Cluster of differentiation; EPCs: Endothelial progenitor cells; ECs: Endothelial cells; NK: Natural killer.

ectopic bone formation[28], underlining the importance of the hematoma in the bone forming process.

The initial pro-inflammatory phase is required to attract progenitor cells, *e.g.*, EPCs or MSCs, to the site of fracture. MSCs with their manifold immunomodulatory and regenerative properties serve as progenitors for fibroblasts, chondrocytes and osteoblasts involved in the following callus formation. NK-, B-, and T-cells contribute to the effect of MSCs during this phase of fracture healing. Osteoblasts are the bone forming cells, producing collagen I that serves as the matrix for mineralization. In a mouse osteotomy model lacking mature T- and B-cells the mineralization process was accelerated[29]. However, in these animals an imbalance between collagen subunits I A1 and I A2 was observed. Histologically, the resulting arrangement of collagen I fibers appeared porous and osteoblasts showed an altered distribution within the fracture callus. These changes within the mineralization process were attributed to the lack of T-cells, using mouse models with either B- or T-cell deficiency[29]. Thus, T cells guide osteoblast distribution and the deposition of collagen I during callus formation. Later, homeostasis of bone is mediated mainly by the interaction between osteoblasts and osteoclasts, which derive from monocytes. The balance between these cell types is tightly regulated by the response of the respective progenitors, as well as T-cells acting during the remodeling phase of fracture healing[29].

The participation of each of the described cell types is required for successful fracture healing. In the following sections, the interplay between different immune cell types and osteoprogenitor cells will be more closely elaborated, starting with the cells of the innate immune response followed by the cells of the adaptive immune response, and the complement system as a mediator between them. Finally, a brief overview on the challenges of preclinical testing of immune-based therapeutic strategies to support fracture healing will be provided.

ROLE OF THE INNATE IMMUNE RESPONSE IN FRACTURE HEALING

Neutrophils

Neutrophils represent an essential part of the innate immune system. Neutrophils derive from common myeloid progenitor cells, which in turn originate from pluripotent hematopoietic stem cells (HSCs) in the bone marrow. The recruitment of

neutrophils to the fracture hematoma may be mediated by released DAMPs originating from injured cells at fracture sites[16]. Mitochondrial particles can act as DAMPs[30], and cytokines such as IL-1α or IL-8 (also known as CXCL8) are known to recruit neutrophils to injury sites[31,32]. With regard to the fracture healing process, neutrophils have been described to strongly induce the initial inflammatory reaction (Figure 2), but their role in the actual healing process is rather underrated.

In vivo, depletion of neutrophils is a common method of investigating their effect on healing processes. In small rodents like mice and rats, neutrophil depletion was shown to delay fracture healing[15], with increased stiffness of the newly formed bone[33], possibly due to reduced MSC infiltration and favored chondrogenic differentiation [34]. This is supported by *in vitro* studies, showing that neutrophils form a kind of extracellular matrix that supports MSC influx into the fracture hematoma[35], but also inhibit matrix formation by MSCs[36]. Furthermore, a negative effect of freshly recruited neutrophils was observed, which induced apoptosis of undifferentiated and differentiated osteogenic cells[37]. Here, a reactive oxygen species (ROS)-dependent mechanism was proposed. This is in contrast to a recent study, showing that neutrophils alter cytokine release by MSCs but not their osteogenic differentiation[38], suggesting that the activation status of the neutrophils is critical in this process.

The observed delay in fracture healing in neutrophil depleted mice was no longer observed when an additional thorax trauma was present, emphasizing the role of neutrophils in systemic inflammatory responses following trauma[15]. Also, the application of neutrophils directly after fracture was reported to improve bone healing in these neutrophil depleted mice. As a possible regulatory mechanism, CCL (C-C-motif chemokine ligand) 2 induced recruitment of monocytes was discussed[39]. CCL2, LL-37 (cathelicidin), AZU1 (azurocidin, also known as cationic antimicrobial protein CAP37 or heparin-binding protein), and other neutrophil secreted factors may pave the way for inflammatory monocytes[40]. Therefore, an indirect role of neutrophils in the healing process is possible, as monocytes are known to support healing by a CCL2-dependent mechanism, which gives a differentiation signal to MSCs[41].

Further investigations suggest that neutrophils may support angiogenesis and revascularization during the healing process, by releasing factors *e.g.*, VEGF (vascular endothelial growth factor) and Ang1 (Angiopoietin 1), inducing inflammatory angiogenesis[42,43]. In addition, neutrophils were shown to release MMP9 (matrix metalloproteinase 9) in response to VEGF, inducing angiogenesis in hypoxic tissue[44].

TNF-α release from neutrophils was also suggested to be a possible mechanism by which neutrophils accelerate wound healing[45,46] and lead to early strength of surgical incisions[47]. However, the role of MSCs was not investigated in these studies. In MSCs, TNF-α was reported to enhance migration[48] but also to inhibit osteogenesis [49,50]. Thus, the direct effect of neutrophils on MSC development remains controversial.

The neutrophil to lymphocyte ratio and neutrophil counts were established as tools to predict trauma outcome and as markers for complication development. In most studies, high circulating neutrophil counts were associated with a higher risk of complications or postoperative mortality[51-55]. Furthermore, a higher neutrophil count in the blood of trauma patients was identified as a predictor for the development of delayed bone healing[56]. In addition, it was shown that neutrophil depletion reduced sepsis development after chest trauma in mice[57]. A variety of studies suggest that neutrophils play a major role in severe complications after trauma such as acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS), MODS (multi-organ dysfunction syndrome), or ischemia-reperfusion damage[57,58]. MSCs were suggested as a possible tool to reduce/prevent tissue damage in these situations. MSCs reduce apoptosis of neutrophils *in vitro*[59] and cause reduced attachment of neutrophils to endothelial cells[60]. The application of MSCs improved phagocytosis in a bacterial sepsis model in mice[61], reduced infiltration of neutrophils into the liver[62], and consequently improved survival in both studies. Furthermore, MSCs reduced inflammation, neutrophil infiltration, and the release of tissue-harming factors by these cells in an injured gut model or a vasculitis model[63,64]. ROS release was either increased[65,66] or reduced[67] depending on the study, but in general, there is consensus that MSCs seem to balance the neutrophil response.

Apart from strong induction of overall inflammation, neutrophils have another defense mechanism, called NETs[68,69]. Consisting of released chromatin covered with antimicrobial peptides and proteases, NETs are strongly bactericidal[68]. There is evidence that NETs are formed in response to trauma[70,71]. However, a general pathologic role of NET formation in healing processes has not yet proven. Never-

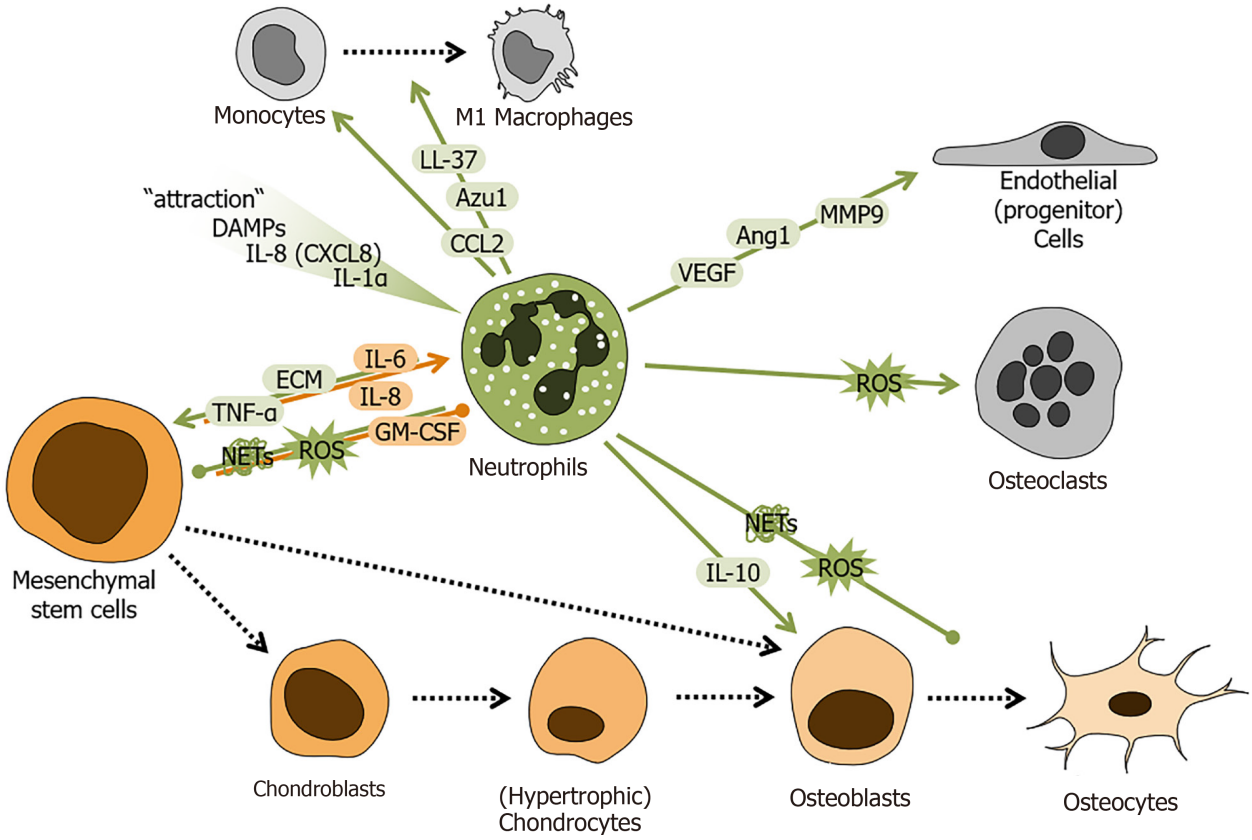

Figure 2 Schematic overview of the regulatory role of neutrophils during fracture healing. Factors secreted/released from neutrophils and mesenchymal stem cells (MSCs) regulate cellular responses during fracture healing. Neutrophil-derived factors are marked in green. MSC-derived factors are marked in orange. Local increase in danger associated molecular patterns, interleukins 8 (IL-8 or CXCL8) and 1 alpha (IL-1α) attract neutrophils to the fracture site. There neutrophils secrete /release factors *e.g.* tumor necrosis factor alpha, IL-10, C-C-motif chemokine ligand 2, azurocidin 1, cathelicidin (LL-37), vascular endothelial growth factor, angiopoietin 1, matrix metalloproteinase 9, reactive oxygen species, neutrophil extracellular traps, and extracellular matrix components to interact with other cells at the site of fracture. MSCs secrete/release factors *e.g.*, granulocyte-macrophage colony-stimulating factor, IL-6 and 8 to affect neutrophils. Colored arrows depict stimulation and blunt end lines inhibition. Dashed black arrows indicate differentiation processes. DAMPs: Danger associated molecular patterns; IL: Interleukin; TNF-α: Tumor necrosis factor alpha; CCL2: C-C-motif chemokine ligand 2; CXCL: C-X-C motif chemokine ligand; Azu1: Azurocidin 1; VEGF: Vascular endothelial growth factor; Ang1: Angiopoietin 1; MMP9: Matrix metalloproteinase 9; ROS: Reactive oxygen species; NETs: Neutrophil extracellular traps; ECM: Extracellular matrix; GM-CSF: Granulocyte-macrophage colony-stimulating factor.

theless, overshooting NET formation can lead to pathological[72] or delayed healing [73]. Furthermore, NETs are known to be toxic to epithelial and endothelial cells[74, 75]; thus, a toxic effect on MSCs is feasible. However, one study showed reduced NET formation and increased survival in a mouse model of lipopolysaccharide (LPS)-induced acute lung injury due to MSC application[76]. *In vitro* stimulation of neutrophils with MSC-conditioned medium increased NET formation, although the overall response was delayed[77].

Another aspect that needs to be considered when discussing neutrophils and trauma is neutrophil phenotypes. Neutrophils are directly affected by trauma. They exert reduced reactivity to stimuli after trauma like bacterial infections[78] or fMLP (N-formylmethionyl-leucyl-phenylalanine)[79-81]. Reduced chemotaxis can be observed to different cytokines after lung trauma[70]. Surface markers [*e.g.*, cluster of differentiation (CD) 62 and 11b, or L-Selectin] and receptors (*e.g.*, CXCR1, CXCR2, or FcγRII, also known as CD32) are altered after trauma and thus also influence neutrophils reaction to other stimuli (reviewed in [78]). Recently, the categorization of neutrophils into "N1" and "N2" phenotypes, similar to monocyte/macrophage and T-cell types, was proposed. Neutrophils were divided into inflammatory (N1) and anti-inflammatory (N2)[82]. Two recent reviews reported the role of neutrophil phenotypes in septic complications after trauma[83,84]. However, their distinct role in tissue repair and their effect on MSCs remain to be elucidated.

In summary, a clear role of neutrophils in the induction of inflammation and possible complications such as SIRS and ARDS has been identified. Their role in the actual healing process and their effect on MSCs remain largely unclear and different results from studies indicate both positive and negative effects on fracture healing. The analysis of neutrophil phenotypes within these results could help to clarify the role of

neutrophils in fracture healing. An overshooting inflammatory response and NET formation by neutrophils are, however, consistently related to tissue damage and prolonged healing.

Monocytes and macrophages

Monocytes and macrophages also belong to the innate immune system. Like neutrophils, systemic or circulating monocytes and macrophages derive from the common myeloid progenitor cells in the bone marrow. In many organs, including bone, tissue-resident macrophages also exist, which predominantly derive from embryonic macrophages. In bone, these tissue-resident macrophages are named osteomacs. Osteomacs are closely associated with areas of bone formation, forming a canopy-like structure on top of the active osteoblasts[85]. Upon depletion of macrophages in a macrophage-fas-induced apoptosis (MAFIA) mouse model, not only the osteomacs but also the layer of active osteoblasts were lost[86], suggesting that macrophages play an active role in osteoblast mediated bone formation and hence fracture healing[87]. Monocytes infiltrate the injury site, usually within one day following fracture. Depending on the milieu they differentiate into immunogenic M1 macrophages or immunosuppressive M2 macrophages, DCs or later even into bone resorbing osteoclasts (Figure 3). In the early inflammatory phase of fracture healing monocytes are stimulated towards the pro-inflammatory M1 phenotype by inflammatory cytokines and to a lesser extent by bacterial products, such as LPS. These M1 macrophages phagocytose cellular debris and pathogens, produce large amounts of nitric oxide and ROS, and secrete cytokines, *e.g.*, interferon (IFN)-γ, TNF-α, and IL-6. Thus, M1 macrophages contribute to the pro-inflammatory response following fracture. The cytokines secreted by M1 macrophages support homing of MSCs to the site of fracture[85,88]. Transforming growth factor beta (TGF-β) and associated activation of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 4 (NOX4) and focal adhesion kinase seem to play a crucial role in this process[89]. Later, during the granulation phase, the phenotype switches towards anti-inflammatory M2 upon stimulation with IL-4 and IL-13. M2 macrophages, which primarily secrete IL-10, actively support tissue repair during soft and hard callus formation by suppressing inflammation[22,90]. IL-10 production, also associated with tolerogenic DCs and regulatory B-cell function, is critically required for fracture healing and bone health. IL-10$^{-/-}$ mice were reported to develop osteopenia in both cancellous and cortical bone by suppressing new bone formation[91].

In a mouse model of intramembranous bone formation, macrophages were closely associated with woven bone deposition by osteoblasts during all the phases of the healing process[92]. Depletion of macrophages affected deposition of woven bone and impaired healing of the defect in this model. Interestingly, macrophage depletion at the time of injury was more detrimental compared to depletion of macrophages at later stages of fracture healing[92]. This observation was confirmed in a mouse model investigating cancellous bone healing of drill holes[93]. In mouse models of endochondral fracture healing, depletion of macrophages in the early inflammatory phase of healing, resulted in reduced callus size, delayed hard callus formation, and delayed fracture union[22,94].

Some *in vitro* studies have closely investigated the effect of the different macrophage phenotypes on bone formation. Conditioned medium from non-activated J774A.1 murine macrophages increases alkaline phosphatase (ALP) activity in MSCs, an effect mediated by secreted bone morphogenetic protein 2 (BMP2)[41]. This result was confirmed by another study showing that non-activated human monocytes enhance proliferation, ALP activity, and expression of osteocalcin and osteopontin, in human bone marrow derived MSCs (BMMSCs). It was found that these effects were not dependent on cell-cell-contacts but partially dependent on BMP2[95]. When cell-cell-contact is provided, non-activated human monocytes stimulate bone formation by BMMSCs *via* the cytokine oncostatin M (OSM), which is secreted by monocytes directly upon cell-cell-contact with MSCs[96]. There are few studies that suggest that the observed effect is enhanced in M1 macrophages[97-99], and that this effect depends on OSM secreted by LPS challenged macrophages following activation of prostaglandin E2 (PGE2) and cyclooxygenase-2 (COX-2)[99,100].

When investigating the roles of monocytes and macrophages in fracture healing, the individual age has to be considered. Comparing fracture healing in young (3 mo) and old (24 mo) mice, no difference in the amount of macrophages in the fracture callus was observed. However, a constant up-regulation of M1/pro-inflammatory gene expression was observed in the macrophages of old mice. Therefore, preventing the infiltration of macrophages into the fracture site improved healing outcomes in old mice[101,102]. Similarly, when surgical reposition of the fracture is required,

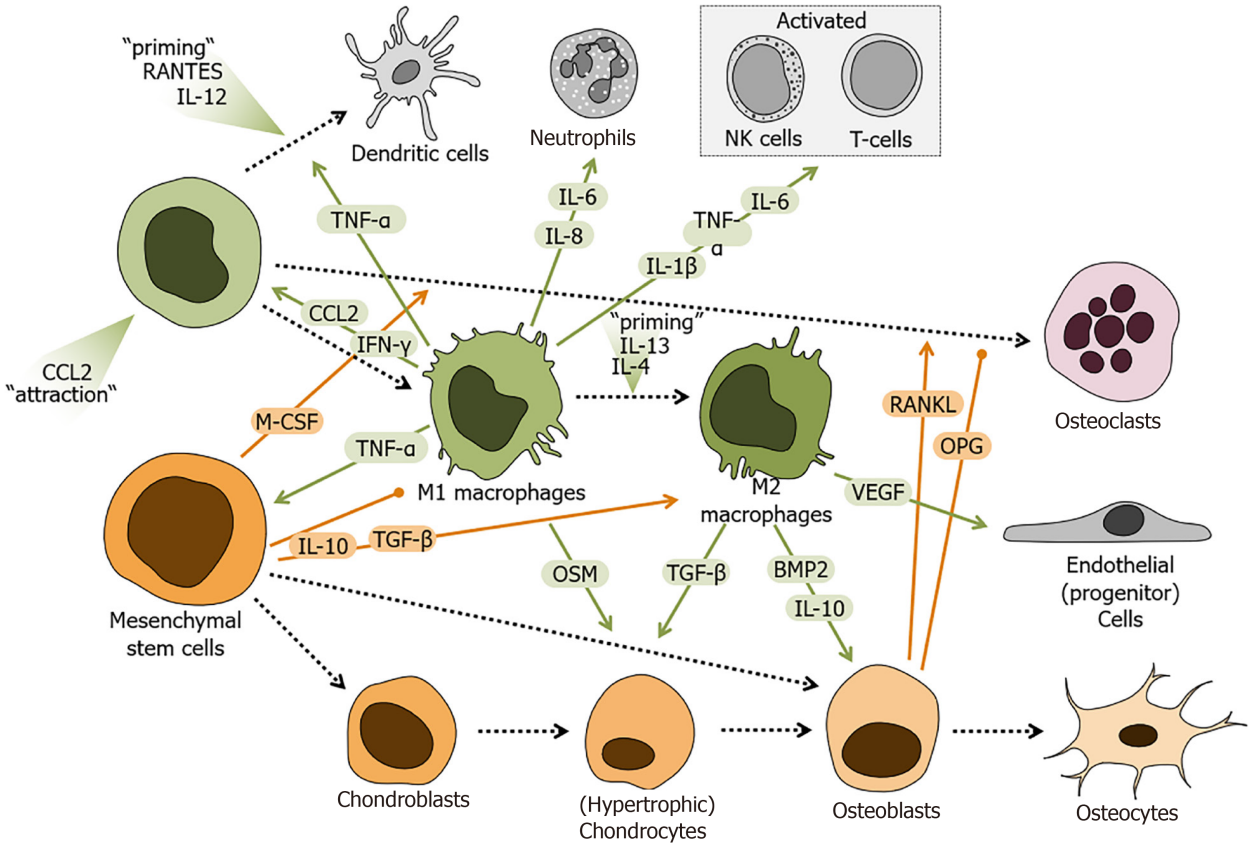

Figure 3 Schematic overview of the regulatory role of monocytes and macrophages during fracture healing. Factors secreted/released from mesenchymal stem cells (MSCs), monocytes, and monocyte-derived cells regulate cellular responses during fracture healing. Factors derived from monocytes and macrophages are marked in green. MSC-derived factors are marked in orange. Local increase in C-C-motif chemokine ligand 2 (CCL2) attract monocytes to the site of fracture, which in the presence of C-C-motif chemokine ligand 5 (RANTES or CCL5) and interleukin 12 (IL-12) differentiate into dendritic cells, in an inflammatory environment are primed towards pro-inflammatory M1 macrophages, or in the presence of IL-4 and IL-13 transform into anti-inflammatory M2 macrophages. Depending on their differentiation state, monocyte-derived cells secrete/release factors *e.g.*, tumor necrosis factor alpha, interferon gamma, CCL2, vascular endothelial growth factor, bone morphogenetic protein 2, transforming growth factor beta (TGF-β), oncostatin M (OSM), IL-1β, IL-6, IL-8, and IL-10 to interact with other cells in the site of fracture. MSCs and MSC-derived cells secrete /release factors *e.g.*, macrophage colony-stimulating factor, IL-10, TGF-β, receptor activator of nuclear factor kappa-B ligand, or its antagonist osteoprotegerin to affect the monocyte derived-cells. Colored arrows depict stimulation and blunt end lines inhibition. Dashed black arrows indicate differentiation processes. CCL2: C-C-motif chemokine ligand 2; IL: Interleukin; TNF-α: Tumor necrosis factor alpha; IFN-γ: Interferon gamma; VEGF: Vascular endothelial growth factor; BMP2: Bone morphogenetic protein 2; TGF-β: Transforming growth factor beta; OSM: Oncostatin M; M-CSF: Macrophage colony-stimulating factor; RANKL: Receptor activator of nuclear factor kappa-B ligand; OPG: Osteoprotegerin.

anesthetics may affect MSC-macrophage interaction. For example, local anesthetics, *e.g.*, lidocaine and bupivacaine, have been reported to directly inhibit secretion of pro-inflammatory cytokines by macrophages, without affecting their viability[103]. Furthermore, these drugs may alter MSC effects in macrophage polarization by attenuating TNF-α and PGE2 secretion[103].

MSCs have been shown to suppress the M1 phenotype in macrophages, including the associated secretion of pro-inflammatory cytokines, in favor of the M2 phenotype with increased production of anti-inflammatory cytokines. This phenotypic switch from M1 to M2 macrophages is possibly mediated by PGE2[104-106], *via* IL1RA (IL-1 receptor alpha), and IL-6[107], and/or by activation of NF-kB (nuclear factor-kappa B) and STAT-3 (signal transducer and activator of transcription 3), which is thought to require IFN-γ mediated indoleamine 2,3-dioxygenase activation[108,109]. Additionally, MSCs seemed to reduce CD86 and major histocompatibility complex (MHC) class II expression in LPS-stimulated macrophages, impairing their immunogenic effects on $CD4^+$ T-cell[104].

MCs

Although, best known for promoting allergic reactions[110], MCs also actively participate in fracture healing[20]. MCs derive from hematopoietic progenitors in the bone marrow and characteristically express CD34 and the surface marker c-Kit (proto-oncogene c-KIT, also known as CD117) that is important for MC growth, differentiation and survival[111]. MC progenitors are released from the bone marrow into the

blood stream and finally mature in the mucosal or connective target tissues dependent on the local environment and growth factor availability[112]. In their large number of secretory granules, MCs store numerous preformed mediators, including cytokines and chemokines (*e.g.*, IL-1β, IL-6, IL-8, TNF-a), histamine, heparin, enzymes (*e.g.*, tryptases, chymases), and growth factors [*e.g.*, VEGF, fibroblast growth factor (FGF), TGF-β]. These mediators can be rapidly released upon activation by cytokines, complement factors, or immunoglobulins (Ig), *i.e.*, through crosslinking of the FceRI (Fc epsilon RI) receptor *via* IgE[113]. MCs interact with many other immune cells *via* these mediators, thus, contributing to both the innate and adaptive immune responses [114]. Furthermore, MCs are capable of *de novo* synthesis of several mediators (*e.g.*, IL-1, IFN-g, RANKL). Many of these MC mediators are known to exert osteo-catabolic (RANKL, TNF-a, histamines) or osteo-anabolic effects [TGF-β, FGF, GM-CSF (granulocyte-macrophage colony-stimulating factor)][115], which is why MCs are supposed to regulate bone metabolism (Figure 4). Confirming this, patients with postmenopausal osteoporosis, rheumatoid arthritis, osteoarthritis, or other inflammatory diseases affecting bone, display increased numbers of MCs in the bone marrow [116-119].

Experimental phenomenological studies showed that MCs may also play a role in bone fracture healing. They are present in the fracture callus, especially in the early soft callus near blood vessels and in the later bony callus in proximity to osteoclasts [120,121]. Recent more mechanistic studies in various MC-deficient mouse models revealed the roles of MCs in fracture-induced inflammation, angiogenesis, as well as in anabolic and catabolic activities during the healing and remodeling process. In more detail, bone healing was delayed in MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice indicated by an impaired transformation of woven bone into lamellar bone, reduced revascularization and increased osteoclast parameters[122]. In MC-deficient $Cpa3^{Cre/+}$ mice, bone repair was also impaired shown by reduced vascularization, bone mineralization and cortical bridging of the fracture callus[123]. However, these mouse models have the drawback that c-Kit is also expressed in osteoclasts and some immune cells, and Cpa3 (carboxypeptidase A3) in basophils and T-cells. Overcoming these limitations, fracture healing was investigated in MC-deficient Mcpt5 Cre R-DTA mice that lack connective tissue MCs without affecting other cell populations[124]. Interestingly, the levels of pro-inflammatory cytokines including IL-6 or CXCL1 were significantly reduced after fracture both systemically and locally in these mice. In addition, chemokine concentrations of KC (keratinocytes-derived chemokine, also known as CXCL1), MIP-2 (macrophage inflammatory protein 2, also known as CXCL2) and granulocyte colony-stimulating factor (G-CSF), known to attract neutrophils, were significantly reduced in MC-deficient mice. This confirmed that fewer neutrophils and macrophages were recruited to the fracture hematoma. These results indicate an important role for MCs in fracture-induced local and systemic inflammation by the release of inflammatory mediators inducing the recruitment and activation of immune cells. Later in healing, callus bone content was increased in MC-deficient Mcpt5 Cre R-DTA mice associated with reduced osteoclast numbers, indicating that MCs may enhance osteoclast activity during callus remodeling. Supporting these findings, *in vitro* analysis further showed that MCs promote osteoclastogenesis *via* granular mediators, especially *via* histamine [124].

In conclusion, MCs are present during the whole fracture healing process and mainly modulate the inflammatory response, vascularization and osteoclastic bone remodeling by using their broad spectrum of mediators. Hence, MCs can also influence MSCs present during the healing process as MSCs are responsive for MC mediators through various receptors, *e.g.*, IL-1 receptor (IL-1R), IL-6R, TNFR (TNF receptor), CXCR1, TGFβRI (TGF-β receptor 1), or bFGFR (basic FGF receptor)[125]. On the other hand, MSCs also secrete factors including TGF-β, VEGF, or IL-6[126], that could modulate the function of MCs, as they express the appropriate receptors (TGFβ R1/2, VEGF receptor, IL-6R)[127]. MSCs are recruited to the injured region following fracture and initiate the repair phase by differentiation into chondrocytes and osteoblasts[128]. A direct interaction of MCs and MSCs during fracture healing has not been identified so far. In physiological bone turnover of MC-deficient $Kit^{W/W\text{-}v}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice, osteoblast parameters were changed probably due to an altered MSC differentiation capacity[129,130]; however, the underlying mechanisms need to be further investigated. *In vitro*, several studies observed effects of MCs on MSCs or *vice versa*. Co-culture experiments revealed that MCs can promote the proliferation of MSCs, but inhibit their differentiation *via* the PDGF (platelet-derived growth factor) pathway[131]. Furthermore, pre-incubation of MSCs with exosomes isolated from MCs induced the migration of MCSs *via* exosomal TGF-β[132]. Pre-treatment of MSCs with MC conditioned medium improved the therapeutic effect of MSCs in atopic

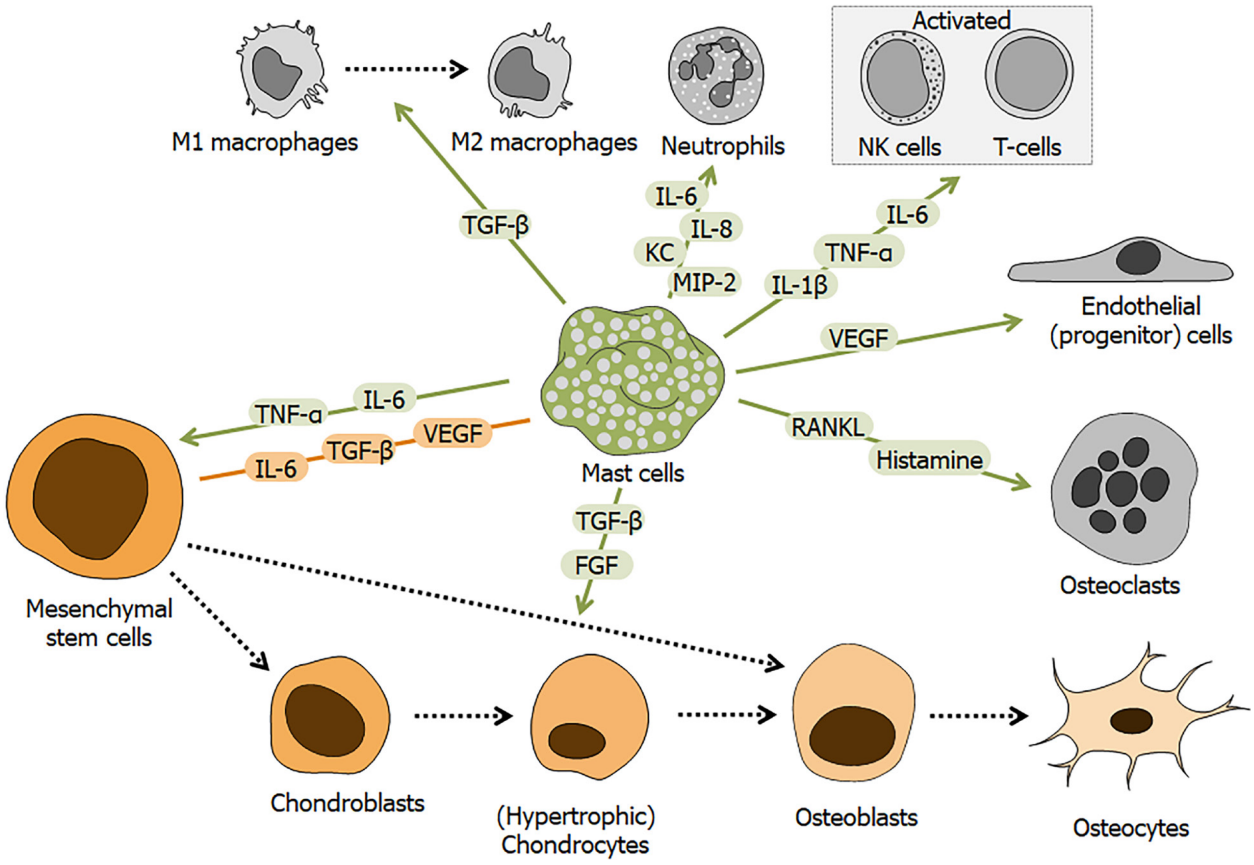

Figure 4 Schematic overview of the regulatory role of mast cells during fracture healing. Cellular responses during fracture healing are orchestrated by factors secreted/released from mast cells and mesenchymal stem cells (MSCs). Factors derived from mast cells are marked in green. MSC-derived factors are marked in orange. Mast cells communicate with other cells during fracture healing by producing and releasing a large variety of factors *e.g.*, tumor necrosis factor alpha, interleukin 1 beta (IL-1β), IL-6, and IL-8, keratinocytes-derived chemokine (KC or CXCL1), macrophage inflammatory protein 2 (MIP-2 or CXCL2), fibroblast growth factors, vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), receptor activator of nuclear factor kappa-B ligand, or histamines. MSCs in turn affect mast cells by secreted factors *e.g.*, IL-6, TGF-β, or VEGF. Colored arrows depict stimulation and blunt end lines inhibition. Dashed black arrows indicate differentiation processes. TNF-α: Tumor necrosis factor alpha; IL: Interleukin; FGF: Fibroblast growth factor; TGF-β: Transforming growth factor beta; VEGF: Vascular endothelial growth factor; RANKL: Receptor activator of nuclear factor kappa-B ligand.

dermatitis in mice[133]. *Vice versa*, MSCs can also influence MCs in a co-culture system, by reducing their degranulation and cytokine production[134]. Moreover, the culture medium of MSCs pre-treated with TNF-a inhibited MC activation and histamine release in a model of allergic conjunctivitis[135]. MSC administration in various inflammatory settings including interstitial cystitis[136], atopic dermatitis [137], intracranial aneurysm[138], osteoarthritis[139], or allergic rhinitis[140], improved disease outcome, *i.e.*, by reducing the number of MCs or their degranulation.

Hence, the effects of MCs on MSCs during bone healing are likely due to influencing migration, proliferation, and differentiation of MSCs. This might be of special interest in conditions of MC accumulation as observed in osteoporosis and probably also during fracture healing in osteoporotic bone or other inflammatory conditions in bone. Moreover, MSCs might also directly influence MC behavior during fracture healing by modulating MC numbers, degranulation, cytokine production and mediator release. In conclusion, more and more specific roles of MCs in fracture healing have been identified in recent years; however, the crosstalk of MCs and MSCs in this context requires further elucidation.

DCs

DCs derive from common myeloid progenitor cells in the bone marrow. DCs are specialized antigen presenting cells (APCs) that can also take up and process antigens, and have the capacity to stimulate resting T-cells in the primary immune response [141]. DCs process phagocytosed antigens into peptides in order to present them to T-cells *via* MHC molecules on their cell surface, which primes T-cells as part of the adaptive immune response[142]. DCs differentiate from monocytes and secrete IL-12, favoring the differentiation of naïve $CD4^+$ T-cells toward T helper type 1 (Th1) cells,

thus, contributing to the pro-inflammatory response required for homing of MSCs. Therefore, DCs are assumed to be active mainly in the early phases of fracture healing (Figure 5). However, the specific roles of DCs in fracture healing are yet to be elucidated. MSCs in turn have been shown to impair the maturation of DCs from monocytes or $CD34^+$ hematopoietic precursors[143]. As a result, fewer pro-inflammatory cytokines were secreted. This MSC mediated inhibition of DC function seems to be dependent on cell-to-cell contacts[144]. Another study suggested that MSCs inhibitory effects on DCs are related to the production of TGF-β and downregulation of DC costimulatory molecules (*e.g.*, CD40, CD80, CD86), thus contributing to the activation of regulatory T-cells (Tregs)[145,146]. Yet another study suggested that MSCs secrete growth-regulated oncogenic chemokines when co-cultured with monocyte-derived DCs, which acquire a myeloid-derived suppressor cell-like phenotype under this condition[147]. Furthermore, MSCs were reported to induce expression of *SOCS1* (suppressor of cytokine signaling 1) in DCs in an IL-6-dependent manner. This way, DCs acquire a tolerogenic phenotype with increased production of IL-10[148].

NK cells

NK cells, as part of the innate immune system, make up approximately 5% to 10% of all lymphocytes within peripheral blood. They derive from common lymphoid progenitor cells, which originate from HSCs in the bone marrow. Upon their primary mode of action, conventional NK cell subsets can be characterized by the expression of surface marker CD56. $CD56^{dim}$ NK cells mainly exert cytotoxic activities against tumor or infected cells *via* a MHC class I dependent recognition mechanism. $CD56^{bright}$ NK cells show an increased cytokine production capacity mainly secreting IFN-γ and TNF-α, thereby, amplifying immune responses[149,150]. NK cells require priming by IL-12, IL-15, IL-18, IL-21, and IFN-α/β prior to activation which underlines that their range of action is widely believed to be within infectious environments, where classically activated NK cells contribute to the Th1 response[151-153].

The role of NK cells during trauma and their interaction with MSCs is not fully understood and seems strongly dependent on the current inflammatory status. After trauma, NK cells are among the first cells to arrive at the site of injury attracted by TNF-α and IL-6[21] (Figure 5). General immune suppression as a response to major trauma also affects NK cells. For example IFN-γ secretion was suppressed, following major trauma, when facing infectious challenges mimicked by *Staphylococcus aureus* [154]. *In vitro*, NK cell activity was shown to be suppressed when incubated with fluids from early fractures or soft tissue injuries[155]. Decreased phosphorylation of mTOR (mechanistic target of rapamycin) and increased CD117 expression were identified as regulators of trauma-induced NK cell dysregulation[156]. To escape NK cell-driven lysis due to generally low MHC class I expression or NK cell mediated harm through massive IFN-γ production, MSCs were shown to be able to adapt within inflammatory environments. For example, MSCs were reported to increase MHC class I expression in response to high IFN-γ levels or increased resistance against cytotoxic NK cells upon Toll-like receptor 3 stimulation[157-159]. MSCs were found to be recruited to non-infectious environments, by CXCL7 secreted by primary unstimulated NK cells[160]. Classically, MSCs are reported to exhibit immune-suppressive properties towards NK cells — they secrete IL-10, TGF-β, and PGE-2, thereby limiting NK cell function and proliferation[161-163]. However, the immune-stimulatory effects of MSCs have also been reported. $CD56^{bright}$ NK cells, primed with IL-12 and IL-18, showed increased secretion of IFN-γ when co-cultured with MSCs without direct cell-cell contact[164]. CCL2 was identified as the main immunomodulatory cytokine in this process. MSCs secreted CCL2 in response to IFN-γ, which primed NK cells for additional IFN-γ release in a positive feedback loop[165]. A recent study showed a time-dependent effect of MSCs on NK cells in the context of infected tissue injury. Shortly after injury (4 h) MSCs induced a pro-inflammatory response in NK cells by stimulating IFN-γ release. However, 24 h post-infection, MSCs induced a senescence-associated NK cell phenotype (SASP) by TGF-β and IL-6 secretion, which was accompanied by a change in the population from $CD56^{bright}$ $CD16^+$ to $CD56^{bright}$ $CD16^-$. SASP NK cells then triggered further IL-6 release, angiogenesis, and MSC proliferation, overall favoring tissue regeneration[166].

In summary, these studies suggest, that there is a tight interplay between NK cells and MSCs during fracture healing. However, the effects strongly depend on the inflammatory status.

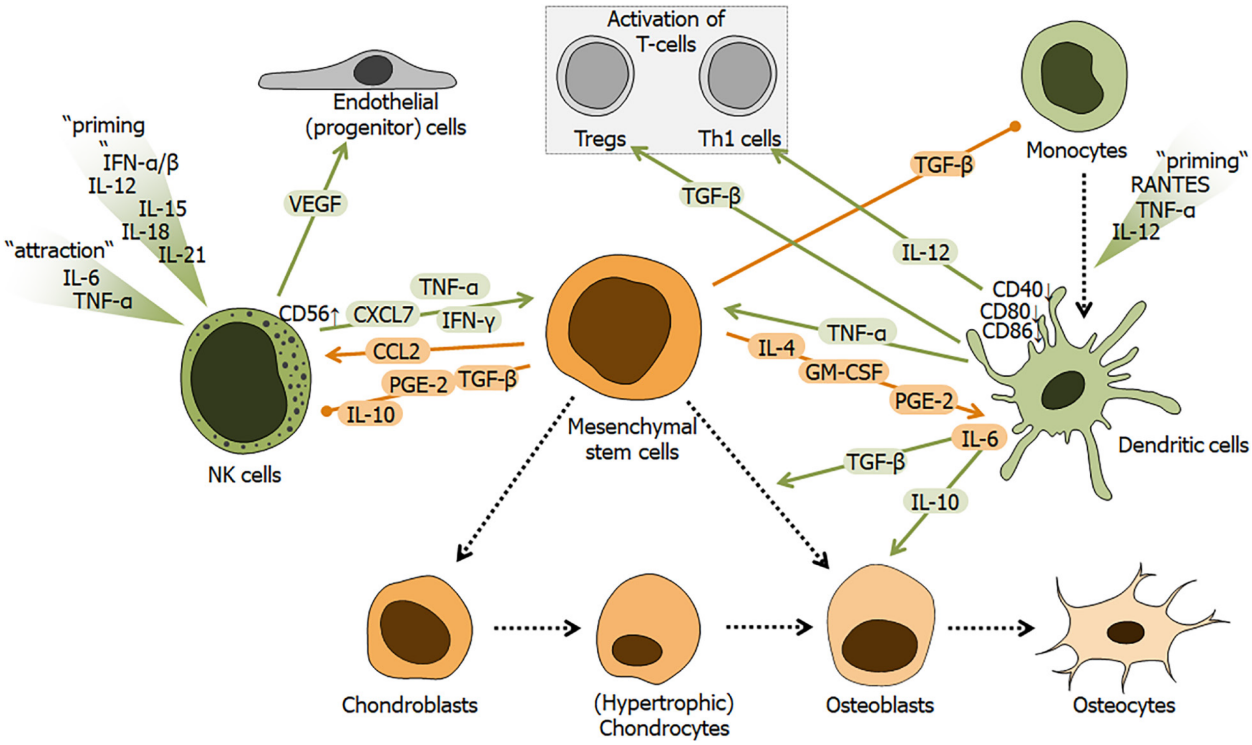

Figure 5 Schematic overview of the regulatory role of dendritic cells and natural killer cells during fracture healing. Fracture healing is affected by factors secreted / released from mesenchymal stem cells (MSCs), natural killer cells (NK cells) or monocyte-derived dendritic cells. Factors derived from NK cells and dendritic cells are marked in green. MSC-derived factors are marked in orange. NK cells are attracted and primed by local increase in factors, *e.g.*, tumor necrosis factor alpha (TNF-α), interferons alpha and beta (IFN-β), and interleukin 6 (IL-6), IL-12, IL-15, IL-18, or IL-21. NK cells, depending on their activation status, secrete factors, *e.g.*, TNF-α, interferon gamma, vascular endothelial growth factor, or pro-platelet basic protein (PPBP or CXCL7). Dendritic cells differentiate from monocytes in the presence of TNF-α, IL-12, and C-C-motif chemokine ligand 5 (RANTES or CCL5). Dendritic cells then secrete factors, *e.g.*, TNF-α, transforming growth factor beta (TGF-β), and IL-10 and IL-12. Both cell types are strongly affected by factors secreted/released from MSCs, *e.g.*, C-C-motif chemokine ligand 2 (CCL2), prostaglandin E2 (PGE2), granulocyte-macrophage colony-stimulating factor, TGF-β, or IL-4, IL-6 and IL-10. Colored arrows depict stimulation and blunt end lines inhibition. Dashed black arrows indicate differentiation processes. CD: Cluster of differentiation; NK: Natural killer; TNF-α: Tumor necrosis factor alpha; IL: Interleukin; IFN-α: Interferon alpha; IFN-γ: Interferon gamma; VEGF: Vascular endothelial growth factor; CCL: C-C-motif chemokine ligand; TGF-β: Transforming growth factor beta; PGE2: Prostaglandin E2; GM-CSF: Granulocyte-macrophage colony-stimulating factor.

ROLE OF THE ADAPTIVE IMMUNE RESPONSE IN FRACTURE HEALING

T-cells

T-cells play crucial roles in the adaptive immune response. They are of hematopoietic origin, as they derive from common lymphoid progenitor cells in the bone marrow. Maintenance of these common lymphoid progenitor cells is strongly dependent on the recently described subset of osteolectin positive MSCs, which can be found in close proximity to the arterioles in the central bone marrow and the endosteum[4]. It has been shown, that these osteolectin positive MSCs are required for T-cell as well as B-cell mediated bacterial clearance following an infection[4]. Using bone regeneration as a model, the effect of the adaptive immune system and more specifically the impact of T-cells has been investigated in order to first understand their role in regenerative processes and to secondly explore the possibility to use immune modulatory strategies to develop new therapeutic approaches. Similar to the cells of the innate immune response, T cells are also involved in many steps of fracture healing[167] (Figure 6). However, their activation status seems to be crucial. As discussed in the introduction, there is a loss of regenerative capacity in mammals starting after birth and upon aging. At birth, the adaptive immune system is still naïve. The number of naïve T-cells decreases with age, changing into effector, effector/central memory and terminally differentiated T-cells in a rate that is dependent on the antigens the individual encounters over time[168]. This highly individual immune aging process is therefore somewhat separated from chronological aging. In order to determine whether immune age influences bone properties, an approach was chosen where one group of mice was aged under sterile (specific pathogen free) conditions while a complementary group was housed under conditions where they encountered environmental pathogens. While the first group remained more or less immunologically naïve over the period of

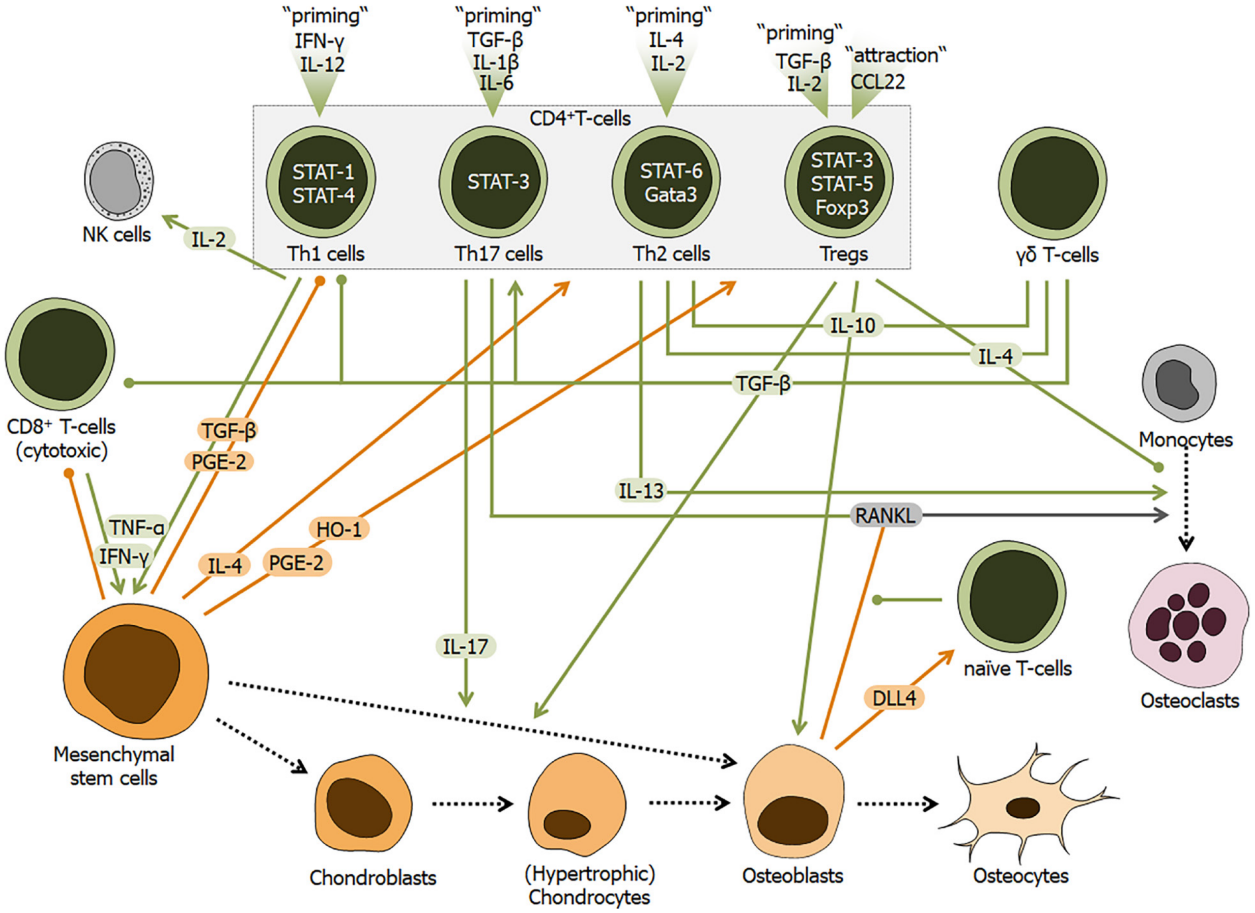

Figure 6 Schematic overview of the regulatory role of T-cells during fracture healing. Fracture healing is affected by interactions between mesenchymal stem cells (MSCs) and T-cells. However, the effect strongly depends on the activation or differentiation status of the T-cells, which is in $CD4^+$ T-cells strongly dependent on activation of signal transducer and activator of transcription (STAT) signaling. Th1 cells are primed by interferon gamma (IFN-γ) and interleukin 12 (IL-12), which activate STAT-1 and STAT4 signaling in these cells. Th1 cells then secrete factors *e.g.*, tumor necrosis factor alpha (TNF-α), IFN-γ, or IL-2. Th17 cells are primed by transforming growth factor beta (TGF-β), IL-1β and IL-6, which activate STAT3 signaling in the cells. Th17 cells then secrete factors *e.g.*, IL-17 and receptor activator of nuclear factor kappa-B ligand (RANKL). Th2 cells, characterized by activated STAT-6 and GATA3 (GATA Binding Protein 3) signaling, are primed by IL-2 and IL-4, and secrete factors *e.g.*, IL-4, IL-10 and IL-13. Regulatory T cells (Tregs) are attracted and primed by factors, *e.g.*, C-C-motif chemokine ligand 22 (CCL22), TGF-β, and IL-2, which activate forkhead box P3, STAT-3 and STAT-5 signaling in these cells. Tregs then secrete factors *e.g.*, IL-4, IL-10, and TGF-β, to regulate osteoblast and osteoclast function, but also activation of T-cells. The same factors (IL-4, IL-10, and TGF-β) are also released by γδ T-cells. $CD8^+$ cytotoxic T-cells enhance the pro-inflammatory reaction by releasing factors, *e.g.*, TNF-α and IFN-γ. The different T-cell subsets, are influenced by MSCs and osteoblasts, which secrete / release factors, *e.g.* TGF-β, IL-4, prostaglandin E2, heme oxygenase 1 (HO-1), RANKL, or delta like ligand 4. Colored arrows depict stimulation and blunt end lines inhibition. Dashed black arrows indicate differentiation processes. CD: Cluster of differentiation; NK: Natural killer; TNF-α: Tumor necrosis factor alpha; IL: Interleukin; IFN: Interferon; CCL: C-C-motif chemokine ligand; TGF-β: Transforming growth factor beta; PGE2: Prostaglandin E2; RANKL: Receptor activator of nuclear factor kappa-B ligand; STAT: Signal transducer and activator of transcription; Tregs: Regulatory T cells; Foxp3: Forkhead box P3; DLL4: Delta like ligand 4.

two years, the second group gained immune experience, developing central and effector memory cells[169]. Comparing bone parameters from these two groups of mice showed a stiffer and more brittle bone in animals with an experienced/aged immune system[169]. This is an indicator of the negative effect the change from the naïve immune status towards an experienced immune status, with central and effector memory T-cells and terminally differentiated T-cells, has on bone. Also the lack of T-cells, especially naïve T-cells, results in lower bone quality and delayed bone healing [169,170]. The above mentioned osteolectin positive MSCs, required for maintenance of the common lymphoid progenitor cells and adequate T-cell response, have been reported to decrease in number with age[4,5]. As the number of naïve T-cells also decreases with age, a direct correlation between these two cell types may be feasible. However, the effects of the MSCs on the T-cells seem to be strongly dependent on the cells activation status. It has been proposed that MSCs cause a downregulation of Fas receptor and Fas ligand on T-cell surface and thus, may rescue T-cells from activation-induced cell death[171]. TGF-β and hepatocyte growth factor, secreted by MSCs, have been identified to be soluble mediators suppressing T-cell proliferation, a process that can be augmented by cell-cell-contacts between the two cell types[172]. An immune

composition with high levels of effector memory and terminally differentiated T-cells could thus be an indicator of delayed or disturbed bone healing. Indeed, a clinical study showed that delayed bone healing occurred in patients with high levels of terminally differentiated $CD8^+$ T-cells[170]. Thus, high percentages of terminally differentiated $CD8^+$ T-cells in peripheral blood could represent a biomarker for delayed healing that could easily be identified within one hour after a patient is hospitalized with a bone injury, opening possibilities for early intervention[170]. Amazingly, high percentages of these effector T-cells in peripheral blood or within the fracture hematoma, were not a result of the injury, but a result of antigen exposure over time.

Better characterization of the different T-cell subsets during bone healing, may shed light on their role in the healing process. Recently, IL-17 producing γδ T-cells, which are present in the fracture during callus formation, have been identified to promote bone healing[173]. However, T-cell differentiation is also critically dependent on osteoblasts, which produce notch ligand DLL4 (delta like ligand 4), the key regulator for this process[174]. Thus, sepsis-induced ablation of osteoblasts contributes to an immune deficiency[175].

Tregs, described to play a pivotal role in peripheral immune tolerance, are able to modulate both the innate and adaptive immune responses. Heme oxygenase-1 (HO-1) is a key contributor to MSC-mediated suppression of allo-activated T-cells, and induction of Tregs. For example, MSCs induce, in a HO-1-dependent fashion, IL-10^+ Tr1 (T regulatory type 1) and TGF-β^+ Th3 Treg-subsets in allo- and T-cell receptor-activated lymphocytes[176]. Furthermore, HO-1 facilitates MSCs to induce Tregs from naïve T-cells and promote their proliferation[176]. Tregs have the ability to alter and kill target cells such as APCs and effector T-cells. Furthermore, they may influence inflammatory cytokine environments and metabolic pathways[177]. Thus, these cells are required for maintenance of self-tolerance, or preventing excessive inflammation and autoimmune diseases. In the setting of trauma, Tregs become prominent when the granulation tissue is formed and remain at the fracture site until remodeling starts. They contribute to the specific release of anti-inflammatory cytokines such as IL-10, inducing a shift towards a Th2 lymphocyte-mediated response and/or lymphocyte anergy, and thus, to profound (post-traumatic) immunosuppression[178,179]. Later during fracture healing, Tregs are thought to control osteoblast and osteoclast function.

In vivo, the number of Tregs in peripheral blood is inversely correlated to serum markers of bone resorption, not only in rheumatoid arthritis patients but also in healthy controls, suggesting that Tregs control bone destruction[180]. Increasing numbers of Tregs improved clinical signs of rheumatoid arthritis and suppressed local and systemic bone destruction[180]. Furthermore, the suppressive effects of Tregs on osteoclast differentiation were confirmed *in vitro*[180]. It was suggested that enhancing the activity of Tregs may beneficially influence the treatment of inflammation-induced bone loss observed in rheumatoid arthritis. Yet, the effects and regulatory mechanisms of Tregs on osteoclastogenesis were investigated only in a limited number of studies. In a monocyte and Tregs co-culture system, Tregs inhibited osteoclast differentiation and reduced the resorbed areas[181]. The authors have shown that this suppression of osteoclast differentiation was cytokine-dependent, as osteoclast differentiation was blocked by anti-TGF-β or anti-IL-4 antibody treatment. As no direct cell-to-cell contacts were required to inhibit osteoclast function by Tregs, TGF-β and IL-4 may represent the key cytokines for this suppressive function of Tregs[181].

RANKL promoting the differentiation of bone-resorbing osteoclasts is thought to negatively impact bone healing, but is also active in T-cell differentiation and proliferation[1]. In a study, patients with isolated closed tibial fracture were subdivided into normal healing and delayed healing groups, based on their healing progression. In these patients, CD45RA-CD62L-effector memory cells most effectively suppressed RANKL. These cells were present at lower frequencies and with functional impairment in patients with delayed healing[182]. Hence, bone-resorbing osteoclast formation may be favored in these patients, suggesting a possible mechanism for delayed bone healing[182]. Another study supported the findings that multiple reductions in Tregs function in delayed healing patients could produce long-lasting consequences in the bone fracture healing process[183].

It has become evident that tissue destruction is associated with a decrease in local regulatory processes, including a decrease in forkhead box P3 (*Foxp3*)-expressing Tregs. CCL22 is a known chemoattractant for Tregs. With the help of a controlled release system, composed of a degradable polymer with a proven track record of clinical translation, poly(lactic-co-glycolic) acid, capable of generating a steady release of CCL22 from a point source effectively recruited Tregs to the site of injection[184].

Upon administration of CCL22 in murine experimental periodontitis, increases in Treg-associated anti-inflammatory molecules, a decrease in pro-inflammatory cytokines, and a marked reduction in alveolar bone resorption were observed[184]. In addition, the application of CCL22 reduced clinical measures of inflammation and improved alveolar bone loss in a ligature-induced periodontitis in beagle dogs[184]. Thus, Tregs recruited to the site of injury by CCL22 are associated with a decrease in bone resorption by reducing inflammation. STAT-3 as a key signaling protein in the skeletal and immune system, and may be a key regulator in this process[185]. The study provides evidence that STAT-3 enhances Tregs-mediated suppression of counteracting inflammation, suggesting that STAT-3 could be used as a prognostic marker to identify patients at risk of developing delayed union or nonunion[185].

In another study, systemic infusion of MSCs improved cell-based bone regeneration *via* upregulation of Tregs[186]. In this study, the immunomodulatory function of BMMSCs was proven *in vitro*. Systemic infusion of these BMMSCs significantly improved cell-based repair of critical-sized calvarial defects in a murine model[186]. In the implantation sites, IFN-γ and TNF-α levels were reduced *via* upregulation of Tregs, resulting in marked enhancement of cell-based bone regeneration, but with only limited contribution of BMMSC homing[186].

Apparently, Tregs also contribute to impaired bone healing induced by local accumulation of $CD8^+$ effector T-cells (T_{EFF}). The endogenous regeneration is impaired by increasing the primary "useful" inflammation toward a damaging level with Tregs regulating the pro-inflammatory reaction to enhance healing[187]. The study provided evidence that $CD4^+$ Tregs might counteract undesired effects of $CD8^+$ T_{EFF}, as the healing outcome was improved by an adoptive Tregs therapy[187]. The data from the mouse osteotomy model were supported by clinical data showing that patients with impaired fracture healing have demonstrated higher T_{EFF}/Tregs ratios compared to uneventful healers[187]. These findings demonstrated the key-role of a balanced T_{EFF} /Tregs response following injury required for successful bone regeneration[187].

Although more and more studies show possible roles of the adaptive immune system in bone healing, the underlying mechanisms and involved cell types are still unclear and remain to be elucidated in further studies.

B-cells

Like T-cells, antibody producing B-cells belong to the adaptive immune response. They also differentiate from common lymphoid progenitor cells, which are derived from HSCs in the bone marrow. MSCs support the development of T- and B-cells from HSCs by soluble factors and cell-cell-contacts[188]. Both cell types infiltrate the fracture callus in a two-waved fashion (Figure 7). Interestingly, the number of B-cells seemed to exceed the T-cells during the fracture healing process, where B-cells progressively underwent effector maturation[189]. Early during callus formation B-cells have been described to undergo direct cell-cell-contact with osteoprogenitor cells, presumably regulating their differentiation. However, as described above, the lack of mature T- and B-cells accelerated the formation of the mineralized matrix in a mouse osteotomy model[29]. However, the observed changes within the mineralized matrix where attributed to the lack of T-cells, using mouse models with either B- or T-cell deficiency[29]. Another study suggested, that B-cells regulatory function is required for successful bone healing, as in patients with delayed healing of tibia fractures B-cells seemed to lose their capability to produce IL-10 with time[190]. Another study even suggested, that the initial expression of IL-10 by B-cells is diminished in patients developing a non-union[191]. During the healing process, these antibody and IL-10 producing $CD27^+$ B-cells effectively suppressed IFN-γ, TNF-α, and IL-2 expression in $CD4^+$ T-cells, as well as IFN-γ and TNF-α expression in $CD8^+$ T-cells in a Foxp3 dependent manner[191]. Likewise, *in vitro* $CD19^+CD27^{bright}$ B-cells suppressed proliferation of $CD4^+$ T-cells and enhanced Foxp3 expression in Tregs. However, the mechanism was not dependent on IL-10 but TGF-β and direct cell-cell-contact[192]. Furthermore, $CD19^+CD27^{bright}$ B-cells were reported to reduce the numbers of pro-inflammatory Th17 cells, independently of cell-cell-contacts[193]. This indicates that B-cells have crucial immunomodulatory roles during fracture healing.

However, B-cells may also affect bone cells and *vice versa*. For example, in rheumatoid arthritis patients, B-cells have been reported to suppress osteogenesis *via* TNF-α and CCL3[194]. Similarly, B-cells inhibited osteoblast maturation when challenged with G-CSF during homing of hematopoietic stem and progenitor cells. Simultaneous activation of osteoclasts, however, was attributed to T-cells[195]. Interestingly, $Rag1^{-/-}$ mice displayed higher than normal levels of osteoclasts, although lacking T- and B-cells[196]. As a possible explanation for this, a response to the elevated osteoblast function was suggested. In turn, MSCs may inhibit proliferation, activation, and

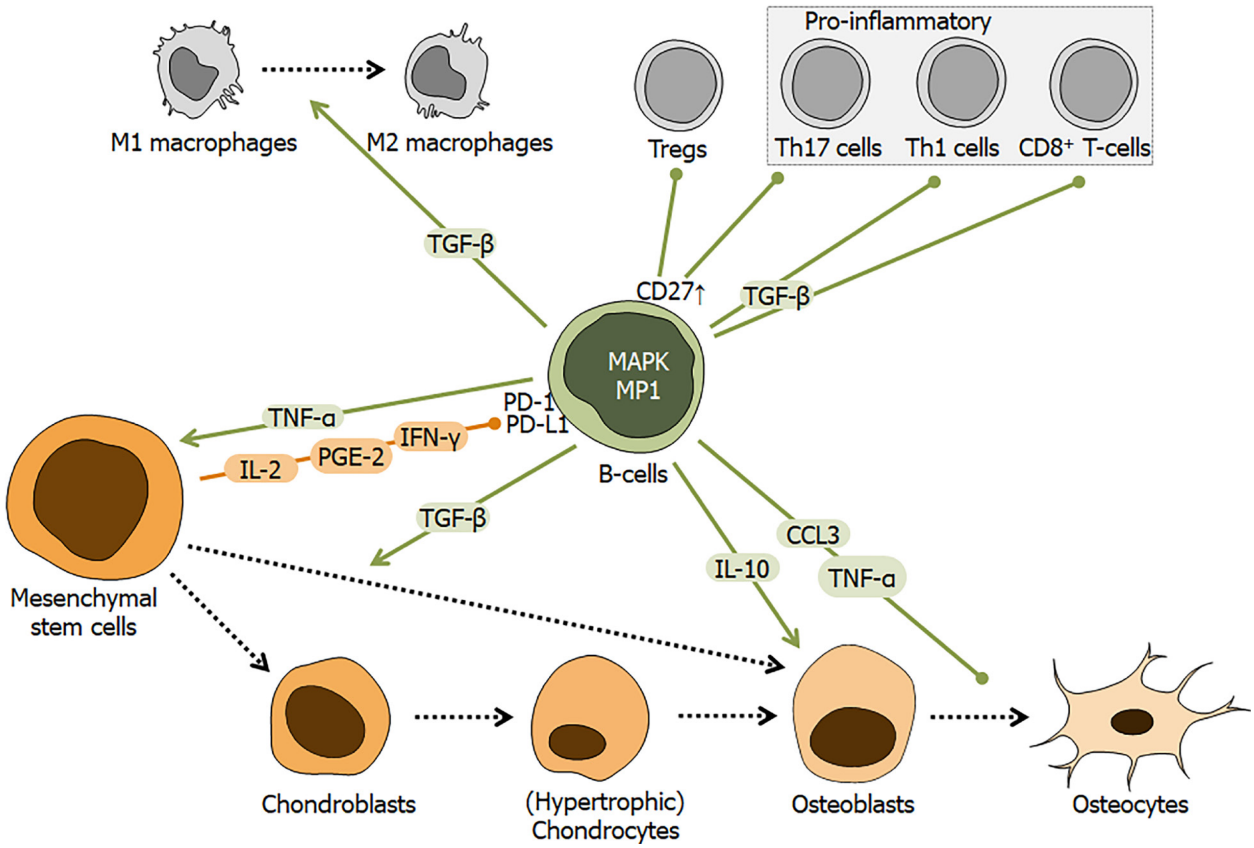

Figure 7 Schematic overview of the regulatory role of B-cells during fracture healing. Fracture healing is affected by interactions between mesenchymal stem cells (MSCs) and B-cells. MSCs may affect B-cells by factors, *e.g.*, interleukin 2 (IL-2), prostaglandin E2, and interferon gamma (IFN-γ), which interact with the programmed cell death 1 receptor and its ligand. Resulting in activation of mitogen-activated protein kinase and its downstream target MEK partner 1, induces B-cells to secrete/release factors, *e.g.*, tumor necrosis factor alpha, transforming growth factor beta, IL-10, and C-C-motif chemokine ligand 3. Colored arrows depict stimulation and blunt end lines inhibition. Dashed black arrows indicate differentiation processes. CD: Cluster of differentiation; TNF-α: Tumor necrosis factor alpha; IL: Interleukin; IFN: Interferon; CCL: C-C-motif chemokine ligand; TGF-β: Transforming growth factor beta; PGE2: Prostaglandin E2; Tregs: Regulatory T cells; MAPK: Mitogen-activated protein kinase; PD-1: Programmed cell death 1; PD-L1: Programmed cell death ligand 1; MP1: MEK partner 1.

antibody secretion of B-cells, possibly by altering MAPK (mitogen-activated protein kinase) activity[197]. It is assumed that MSCs inhibit B-cell proliferation by secreted factors inducing cell cycle arrest in the G0/G1 phase[198], and preventing B-cells maturation by inducing expression of maturation protein-1[199]. Furthermore, MSCs may suppress both B- and T-cell activation by secreted IFN-γ and cell-to-cell contact *via* programmed cell death 1 receptor (PD-1) and its ligand (PD-L1)[200]. *In vitro*, however, contradictory data exist, showing that MSCs may promote the proliferation and differentiation of B-cells[201].

COMPLEMENT SYSTEM

The complement system represents the major fluid phase innate immune surveillance system[202]. As a protein cascade, the complement system contains multiple serine proteases, which can be activated by different pathways early after trauma and during systemic inflammation[202,203]. The generated complement activation products can function as anaphylatoxins (C3a, C5a), opsonins (C3b), or membrane attack complexes (MAC formed by C5b-9) all of which help sensing and clearing of tissue debris, damaged cells, and pathogens after trauma[204]. However, if excessively produced or out of control by suppressed complement regulatory proteins (CRegs), activated complement may also reveal harmful features for the host[203].

On a cellular level, multipotent MSCs are critically involved in healing processes after tissue damage and bone fracture. A hypothesis-free global transcriptional analysis of the bone fracture region post trauma suggested, that several complement and coagulation factors are significantly upregulated at the fracture site[205]. Focusing on MSCs, it is well established that these cells express several key complement receptors (*e.g.*, C3aR or C5a receptors) and membrane-bound CRegs (*e.g.*, CD35, 46, 55,

and 59) all of which play an important role in the concerted recruitment of leukocytes and subsequent induction of repair processes[206]. Trauma can also result in systemic complement activation with generation of the central anaphylatoxins C3a and C5a, which can induce all classical signs of inflammation. C3a represents a potent chemoattractant for MSCs[207] and C5a strongly chemo-attracts neutrophils and macrophages. Moreover, opsonisation of MSCs *via* C3b deposition results in subsequent phagocytosis by macrophages. Concerning the terminal pathway, MAC formation on MSCs can lead to cellular lysis and thus impaired regeneration[206,208]. Concerning cell survival, generation of C5a can induce apoptosis of MSCs[209]; whereas, in contrast, it prevents apoptosis in neutrophils *via* enhanced expression of the anti-apoptotic Bcl-xL [210].

The multifaceted modulation of MSCs by complement activation products led to the development of novel therapeutic approaches. Nanoparticles spiked with the C5aR, functioning as a decoy mechanism for excessive C5a, revealed some protective effects for MSCs[211]. Similarly, "painting" MSCs with factor H, a central native complement inhibitor, turned the MSCs resistant against both a complement- and neutrophil-driven attack[212]. Another approach addresses differentiation processes of MSCs: During the differentiation from MSCs to osteoblasts, C5aR upregulation is dependent on regulation of the urokinase receptor (uPAR) and downstream NfκB transcriptional program. Blocking the C5aR impaired osteogenic differentiation, indicating effective immune modulation of the MSC-driven regeneration process by targeted complement inhibition[213]. However, future translational studies need to investigate and prove the efficacy of such a complement-based MSC modulation.

IMMUNE CELL REGULATION A POSSIBILITY FOR PERSONALIZED TREATMENT TO SUPPORT FRACTURE HEALING?

Delayed or impaired fracture healing, which occurs in up to 20% of all fractures[214], and septic complications represent growing challenges in orthopedic and trauma surgery. Currently, failures in bone healing are detected radiologically 4-6 wk after the initial treatment. This considerably prolongs the healing time in patients with healing deficits. The above-mentioned studies describe crucial roles of the innate and adaptive immune system in these processes (Figure 8). Hence, immune scenarios characteristic in patients frequently developing delayed or impaired bone healing, or even septic complications were identified. The alterations in the immune response usually become apparent early in the healing process of a fracture, some even at the time of hospitalization of the patients. This opens new avenues for early interventions. With a tool to stratify patients with higher risks for delayed healing, therapeutic approaches to treat these patients are needed. With a demographically aging population the percentage of elderly patients, with an educated and aged immune system, presenting to the clinic with a fracture will rise. This factor has to be considered when developing and testing new therapeutic strategies based on immune modulation, emphasizing the need for a paradigm shift in animal models. In 2016, Badylak[215] stated that the immune system as a regulator of organ and tissue development and as an orchestrator of the healing process after injury is often neglected in research models. Although, there are rising numbers of studies emphasizing that the immune status of the pre-clinical model could be decisive for the research results[187,216,217], state of the art is to keep animals as clean as possible, *i.e.* most rodent model housing is often either pathogen free or specific pathogen free. In both housing conditions the immune experience is minimal. This is partly attributed to the fact that so far no standardized method has been found to measure or report immune competence or experience in pre-clinical models. Using mice with effector/memory T-cells, kept under housing conditions that allowed environmental pathogens[169,187], an immunomodulatory strategy was tested that enhanced cyclic adenosine monophosphate during the initial phase of bone healing through local application of the prostacyclin analogue Iloprost. Prostacyclin, previously used to treat bone-marrow edema[218,219], reduced the pro-inflammatory reaction of effector memory T-cells *in vitro*, while strengthening the anti-inflammatory reaction of Tregs, one natural counterpart to $CD8^+$ effector cells[220]. In an immune experienced mouse osteotomy model local delayed release of Iloprost significantly enhanced bone healing, while reducing the number of $CD8^+$ T-cells in the early healing phases[220]. In contrast, a previous study reported that Iloprost inhibited bone healing in a rat fracture model[221]. The discrepancy in statements can be explained by the unstabilized fracture in the rat study or the systemic *vs* local application of Iloprost. However, the immediate prostacyclin administration in the rat study probably

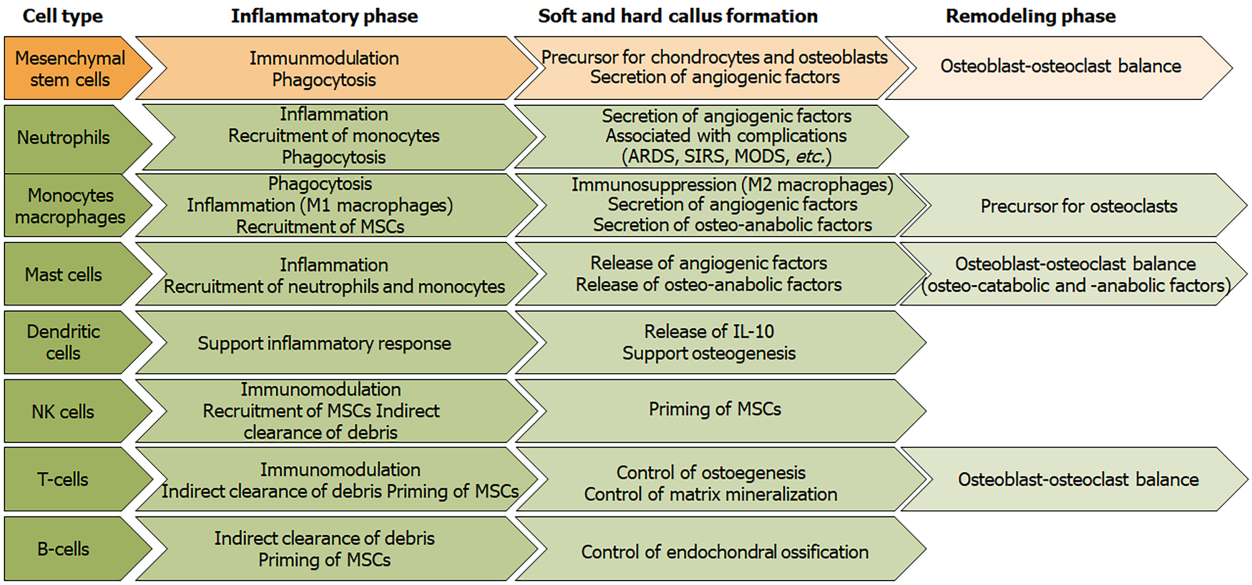

Figure 8 Overview of the roles of immune cells and mesenchymal stem cells during the different phases of fracture healing. NK cells: Natural killer cells; MSCs: Mesenchymal stem cells; ARDS: Acute respiratory distress syndrome; SIRS: Systemic inflammatory response syndrome; MODS: Multi-organ dysfunction syndrome; IL-10: Interleukin 10.

prevented the necessary initial pro-inflammatory reaction and this caused the observed lack in healing. This demonstrates that the complexity of the bone healing process combined with the complexity of the immune system and reaction upon injury demands a very careful strategy when immunomodulation is to be achieved to improve bone healing. Especially as both systems, the bone and the immune system share signaling pathways. This means that by targeting one system one could very well also influence the other. Nevertheless, immunomodulation is a promising future treatment approach to enhance bone healing in patients with an overarching immune reaction to injury that will probably become a personalized therapy option, where the immune composition of the patient has to be taken into account. With bone being a model for regenerative healing, knowledge gained in bone research could become a blueprint to enable scar-less healing in non-regenerative organs in the future.

CONCLUSION

Bone is considered an osteoimmune system based on cooperatively acting bone and immune cells, cohabitating within the bone marrow. The different cell types are highly interdependent, sharing progenitors, mediators, and signaling pathways. MSCs with their manifold immunomodulatory and regenerative properties serve as progenitors for fibroblasts, chondrocytes and osteoblasts during fracture healing. Immune cells of the innate and adaptive immune system influence viability and differentiation capacity of MSCs during this process. Alterations in the immune response usually become apparent early in the fracture healing process, which opens new avenues for early interventions. However, to investigate new therapeutic strategies aiming to balance altered immune responses during fracture healing requires the address of not only the innate but also adaptive immune responses. This raises the need for advanced model systems.

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REVIEW

Regulation of the mesenchymal stem cell fate by interleukin-17: Implications in osteogenic differentiation

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Abstract

Bone regeneration is a tightly regulated process that ensures proper repair and functionality after injury. The delicate balance between bone formation and resorption is governed by cytokines and signaling molecules released during the inflammatory response. Interleukin (IL)-17A, produced in the early phase of inflammation, influences the fate of osteoprogenitors. Due to their inherent capacity to differentiate into osteoblasts, mesenchymal stem/stromal cells (MSCs) contribute to bone healing and regeneration. This review presents an overview of IL-17A signaling and the leading cellular and molecular mechanisms by which it regulates the osteogenic differentiation of MSCs. The main findings demonstrating IL-17A's influence on osteoblastogenesis are described. To this end, divergent information exists about the capacity of IL-17A to regulate MSCs' osteogenic fate, depending on the tissue context and target cell type, along with contradictory findings in the same cell types. Therefore, we summarize the data showing both the pro-osteogenic and anti-osteogenic roles of IL-17, which may help in the understanding of IL-17A function in bone repair and regeneration.

Key Words: Interleukin-17; Mesenchymal stem cells; Osteoblast; Bone; Osteogenesis; Inflammation

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Core Tip: The immune system closely interacts with the bone system in health and disease. Inflammation plays a strategic role in bone homeostasis and turnover. A pro-inflammatory cytokine interleukin-17 (IL-17) is produced in high amounts after bone damage and can influence mesenchymal stem cells' fate toward early osteoprogenitor cells, either as a pro-osteogenic or an anti-osteogenic factor. Although these divergent IL-17 roles in bone formation are still not well understood, different conditions of the local microenvironment, the extent of inflammation, and the specific nature and stage of osteoprogenitor cells can influence the response to this cytokine, affecting the final cell differentiation outcome.

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INTRODUCTION

The interleukin-17 (IL-17) cytokine was first described in 1993 as a cytotoxic T lymphocyte antigen 8 (CTLA8) and was, subsequently, in 1995, reported to share 57% of its sequence homology with the herpes virus saimiri gene 13 (HVS13). Both HVS13 and CTLA8 were shown to costimulate T-cell proliferation by binding to a novel cytokine receptor and were named IL-17, vIL-17, and IL-17R, respectively[1,2]. Later on, Park *et al*[3] defined the cellular requirements for the differentiation of naïve CD4 T-cells into effector T helper cells with the capacity to express and secrete IL-17. This resulted in the discovery of a new subset of T helper cells with proinflammatory functions, referred to as T_H17 cells[4]. Today, IL-17 is recognized as a founding member of the IL-17 family that comprises the cytokines IL-17A (initially named IL-17) through IL-17F, which were discovered *via* screening for homologous genes[5]. By producing IL-17A and IL-17F, T_H17 cells participate in host protection against external pathogens and recruit macrophages and neutrophils to the infection site[6,7].

The dysfunctional regulation of T_H17 may exacerbate the pathogenesis of multiple inflammatory and autoimmune disorders, such as sepsis, pneumonia, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), allograft rejection, and cancer[4,8]. Specifically, the six IL-17 cytokines are secreted glycosylated proteins with molecular weights of about 20-30 kDa and share 20%-50% of their sequence homology with IL-17A. IL-17 family members exhibit a conserved protein C-terminus with two intramolecular disulfide bridges formed by four cysteine residues. Moreover, IL-17s belong to the cystine knot fold superfamily since they dimerize similarly to the nerve growth factor subfamily[8-13]. Furthermore, IL-17A and IL-17F form either homodimers or heterodimers and are co-expressed by linked genes on chromosome 6[6,14,15]. It is well known now that, beyond T_H17 cells, many cell types can produce IL-17, including almost all innate and adaptive immune cells[4,6].

IL-17 cytokines exert their actions by binding to the IL-17 receptor (IL-17R) family, composed of five receptor types (IL-17RA to IL-17RE)[5]. Although its expression level varies widely, IL-17R is expressed ubiquitously and is mainly characterized by a shared SEF/IL-17R (SEFIR) motif in the intracellular domain and two fibronectin III-like regions (FN1 and FN2) within the extracellular environment[5,16,17]. In addition, all IL-17 isoforms bind to IL-17RA, which forms heterocomplexes with other IL-17R subtypes responsible for ligand-binding specificity[8,18].

IL-17A initiates signaling by binding to an IL-17RA/IL-17RC receptor complex. This binding triggers the multifunctional adaptor Act1, a U-box E3 ubiquitin ligase interaction with IL-17R *via* the SEFIR domain (Figure 1). Next, the IL-17 downstream intracellular signaling is activated *via* homotypic interactions between the tumor necrosis factor (TNF) receptor-associated factor (TRAF)6/transforming growth factor β (TGF-β)-activated kinase 1 (TAK1) complex with Act1. This signaling includes nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK) (ERK1,2, p38, and JNK). Similarly, the activated IL-17/IL-17R/Act1 complex can signal *via* the

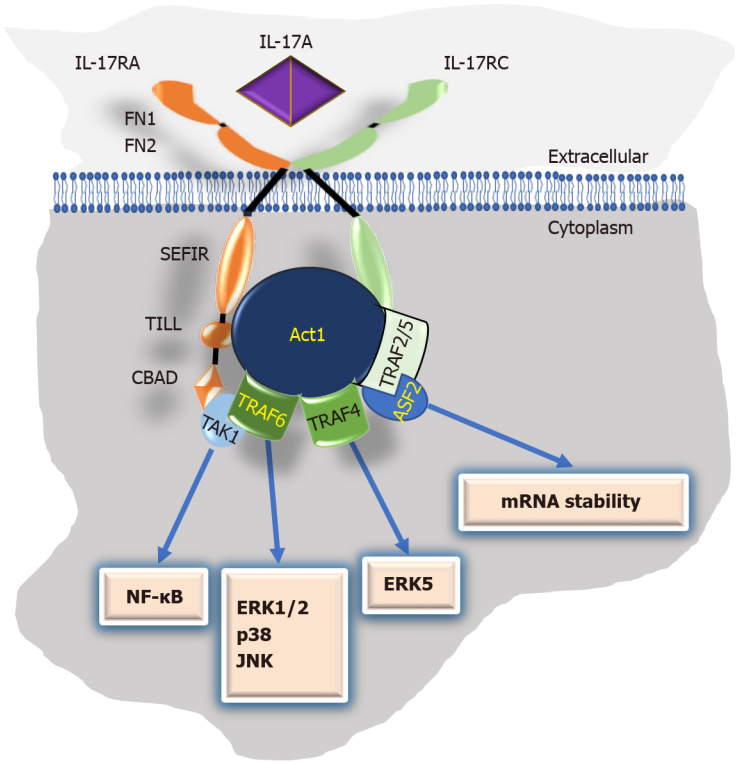

Figure 1 Overview of interleukin-17A signaling. Interleukin (IL)-17A in a dimeric form binds to the cell surface receptor complex comprising IL-17RA and IL-17RC, which triggers the intracellular interaction with the adaptor protein Act1. Then, tumor necrosis factor receptor-associated factor (TRAF) effector proteins associated with Act1: Act1-TRAF6 complex promotes activation of nuclear factor-κB and mitogen-activated protein kinases ERK1,2, p38, and JNK; Act1-TRAF4 complex induces activation of ERK5; and, Act1-TRAF2/5 complex modulates mRNA stability. For more details, see the text. IL-17: Interleukin-17; TRAF: Tumor necrosis factor receptor-associated factor; NF-κB: Nuclear factor-κB.

TRAF4/MEKK3/MEK5/ERK5 axis. Finally, the noncanonical signaling of IL-17A involves a TRAF2/5-human antigen R (HuR)-alternative splicing factor (ASF or SF2) cascade that results in the control of mRNA stability of IL-17-targeted inflammatory cytokine and chemokine genes[5,18-20].

Mesenchymal stem/stromal cells (MSCs) are multipotent stromal cells that were first described in the bone marrow (BM) regarding their capacity to support hematopoiesis after heterotopic transplantation in a nude mouse model[21,22]. MSCs display a spindle fibroblast-like shape and are capable of self-renewal. Moreover, when stimulated, both *in vivo* and *in vitro*, they can differentiate into several mesodermal cell types[23,24]. Moreover, it is now well known that MSCs can also differentiate into nonmesodermal lineages, such as hepatocytes, neurons, and pancreatic cells, among others[24].

MSCs are defined by standardized criteria for their identification and characterization. In 2006, the International Society for Cellular Therapy proposed a set of minimum standards to characterize MSCs: fibroblast-like morphology, plastic adherence, three mesodermal lineage differentiation capacities (adipocytes, osteocytes, and chondrocytes), and specific immunophenotype surface markers[25], whereby more than 95% of MSCs should express CD73, CD90, and CD105. Meanwhile, to avoid hematopoietic cell contamination, leukocyte markers CD45, CD34, CD14 or CD11b, CD19 or CD79α, and HLA-DR should be expressed in less than 2% of the cell population. Recently, additional cell surface markers have been identified that ensure the isolation of clonogenic MSCs such as STRO-1, CD29, CD44, CD106, CD146, and CD27 and epidermal growth factor receptor, insulin-like growth factor (IGF) receptor, and nerve growth factor receptor[26].

MSCs are present in almost all adult tissues[27]. Adipose tissue, BM, and dental tissue are the preferred sources for preclinical and clinical research[24,28,29]. Furthermore, the usage of adult MSCs is not compromised by the biological and ethical concerns that surround their embryonic counterparts. Thus, they can be used as autologous transplants, which has opened up new opportunities for tissue regeneration and bioengineering, as well as for cell-based clinical applications[30-32]. Moreover, when transplanted, MSCs do not manifest tumorigenicity, which is an advantage compared to induced pluripotent stem cells[33,34].

Under homeostatic conditions, MSCs are hypoimmunogenic and capable of evading immune system recognition. In addition, they express low class I major histocompatibility complex (MHC) molecules and lack class II MHC and costimulatory molecule (CD40, CD80, and CD86) expression. These characteristics make MSCs suitable for allogeneic transplantation[35,36]. Likewise, MSCs possess remarkable immunosuppressive, immunomodulatory, and anti-inflammatory functions, accompanied by antimicrobial properties. Thus, MSCs are meaningful candidates to be studied and potentially may be used in therapies for fracture healing and bone regeneration[37-40].

THE BONE STRUCTURE AND FUNCTION

Bone is a supportive tissue contributing to locomotion, soft tissue and vital organ protection, blood pH regulation, and calcium and phosphate homeostasis. It also provides a functional milieu for blood production in the BM and progenitor cell niche formation. In this regard, the bone contains both mesenchymal and hematopoietic cell compartments[41-43].

Bone tissue is mainly composed of two interrelated compartments: (1) Like connective tissue, bone is rich in the extracellular matrix (ECM) and abundant in organic collagen fibers (comprising about 90% of the matrix proteins) and inorganic hydroxyapatite (a naturally occurring mineral significant for bone reinforcement)[43-46]; and (2) The cellular components of bone mainly encompass osteoprogenitors, osteoblasts, osteocytes, and osteoclasts[45,47,48].

Osteoblasts are differentiated cells originating from BM MSCs. Undifferentiated MSCs reside in the periosteum, which covers the bone surface. The osteogenic process occurs in sequential events, including MSC recruitment to bone remodeling areas, followed by cell proliferation and subsequent lineage commitment[49]. In the beginning, MSCs are committed to generating actively proliferating pre-osteoblasts, which at this early stage do not produce ECM proteins. Next, cells cease proliferation and start to secrete type I collagen, proteoglycans, and other noncollagenous proteins. Afterward, the mineralization process occurs with the phosphates released by osteoblast-associated phosphatases, which combine with calcium to form hydroxyapatite crystals. Once a functional ECM is generated, osteoblasts differentiate into osteocytes, long-lived cells with an average half-life of 25 years, embedded within lacunae[49].

Interestingly, osteocytes encompass approximately 90%-95% of bone cells and are recognized as the principal regulators of bone homeostasis since they contribute to bone formation and resorption during bone remodeling. In addition, osteocytes may act as sensors for organic and inorganic molecules during mechanical stimuli to remodel the environment, thus contributing to the proper maintenance of bone tissue functionality[50-55]. On the other hand, osteoclasts are large multinucleated bone-resorbing cells that originate from the fusion of myeloid precursors of the monocyte/macrophage lineage and participate in bone degradation, bone turnover, and remodeling[56,57].

Bone tissue is created by intramembranous ossification or endochondral ossification. In the first place, direct ossification occurs in the neuro and viscerocranium, flat bones, and in part of the clavicle. It is characterized by MSC-derived osteoblast condensation, which causes mature osteoblasts to evolve into osteocytes. Meanwhile, indirect endochondral ossification occurs in long bones, vertebrae, the skull base, and the posterior skull[58]. This process involves MSCs, which initiate the first round of cartilage differentiation and are later replaced by bone tissue, considerably increasing the ability to withstand mechanical compression[59].

Healthy bone is a dynamic organ with a constant balance of fine-tuned bone resorption and new tissue generation. It confers bone's ability to repair itself by continuous skeletal adjustment to mechanical forces in varying environmental conditions[41,43]. Therefore, impairment in cell differentiation can result in different bone pathologies. For example, an imbalance in BM MSC differentiation toward the adipocyte lineage, to the detriment of osteoblast/osteocyte generation, may result in bone mass loss and bone diseases such as osteoporosis[60,61].

Mechanistically, early osteogenesis stages include the expression of hedgehog proteins, Wnt/β-catenin signaling, bone morphogenetic proteins (BMPs), endocrine hormones, epigenetic regulators, cytokines, and growth factors. These events implicate complex processes of finely regulated and timely orchestrated activation of specific transcription factors to express genes that accurately define the osteoblast phenotype [60,62].

Runt-related transcription factor 2/core-binding factor subunit alpha-1 (Runx2/Cbfa1) and downstream osterix (OSX) are crucial for osteoblast differentiation. The absence of either Runx2 or OSX results in the impairment of skeleton mineralization. Moreover, Runx2 is essential for MSC commitment toward the osteogenic lineage[63]. Therefore, Runx2 is expressed early during osteogenesis. However, as the differentiation process advances, Runx2 expression is downregulated, accompanied by upregulation of OSX and β-catenin with further osteoblast maturation[60,62].

Namely, Runx2 contains a runt DNA-binding domain harbored by several enhancers and promoters, including those for the genes encoding alkaline phosphatase (ALP), collagen type 1 (COL1), osteocalcin (OC), and osteopontin (OPN). These proteins contribute to bone matrix generation and osteoblast maturation. These genes are also useful as markers for different osteogenesis stages[64-66]. The time course of events indicates that ALP is an early marker of osteogenic differentiation and mineralization in committed osteoprogenitors. In contrast, more advanced osteogenesis stages implicate COL1, osteoprotegerin (OPG), and osteonectin expression, while OC and OPN are confined mainly to the terminal differentiation phase[62,67-69]. OPG, first characterized and named for its protective role in bone remodeling[70,71], functions as a soluble decoy receptor for the cytokine receptor activator of NF-κB-ligand (RANKL) since it prevents the binding of RANKL to the receptor activator of NF-κB (RANK). Therefore, OPG inhibits osteoclastogenesis and protects bone from excessive osteoclast-mediated resorption[72].

In turn, osteonectin is a binding-calcium glycoprotein implicated in mineralization initiation, promoting mineral crystal formation[73]. Furthermore, OC is vital for bone formation and resorption inhibition[74]. Finally, OPN is an integrin-binding glycoprotein expressed at high levels by osteoblasts at the endosteal surface and regulates bone development and bone mass maintenance[75].

Several signaling factors are involved in the activation of Runx2, including wingless-type (Wnt)/β-catenin, BMPs, TGF-β1, hedgehog, and (Nel)-like protein type 1 (NELL-1)[76,77]. The Wnt/β-catenin signaling pathway may regulate osteoblastogenesis by modulation of MSC commitment to the osteoblastic lineage. The activated Wnt/β-catenin canonical pathway contributes to the induction of osteogenic regulators Runx2, distal-less homeobox 5, and OSX, which notably induces MSCs' progression into mature osteoblasts[43,78-80]. Furthermore, Wnt/β-catenin controls bone resorption by increasing the OPG/RANKL ratio[81,82]. Wnt5a induces noncanonical Wnt signaling pathways, such as the co-repressor complex, through calcium-calmodulin-dependent protein kinase II-TAK1-Nemo-like kinase signaling, to regulate MSC differentiation to osteoblasts by Runx2 induction and inhibition of the adipogenic transcription factor PPARγ expression[83,84].

In addition, a large body of experimental evidence unequivocally demonstrates that BMP signaling causes multipotent mesenchymal cells to differentiate into the osteochondral lineage and regulates the maintenance of postnatal bone and cartilage. The abundance of different types of BMPs varies in response to skeletal requirements. BMP-2, -4, -6, -7, and -9 are of particular importance in bone formation, as they activate BMP-associated Smads (Smad-1, -5, and -8) to induce Runx2 and OSX activation axes, while BMP-3 and BMP-13 present exceptions in the subfamily and act as inhibitors of osteogenic differentiation[85,86]. Moreover, inhibitor of differentiation (ID) proteins, especially ID1 and ID3, are critical effectors of BMP-induced osteoblastogenesis[87].

Furthermore, early-response genes that activate downstream BMP signaling in primary BM-MSCs include Dlx2 and 5. *In vitro* studies demonstrated that Dlx proteins mediate the expression of several osteoblast lineage genes, including Runx2, OSX[88], and osteoactivin, a positive regulator of bone formation, both *in vitro* and *in vivo*[89].

On the other hand, during osteoblastogenesis, inhibitory Smad6 can intracellularly inhibit BMP receptors. Furthermore, BMP-Smad1–Runx2 regulates Smad6 expression, while Smad6 regulates BMP and Runx2 activity in a negative feedback loop[86,90]. Likewise, Noggin, chordin, gremlin, and follistatin, which sequester BMPs and prevent binding to cell surface receptors, regulate BMP function during bone generation[86,88].

Also, systemic hormones, such as parathyroid hormone, glucocorticoids, estrogens, and local growth factors, such as bone TGF-β1/2, IGF, fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor, prostaglandins, and MAPK signaling molecules, regulate MSC osteogenic differentiation[41]. Furthermore, MSC osteoblastogenesis can also be induced *in vitro* by adding a combination of dexamethasone, beta-glycerophosphate, and ascorbic acid to the cell culture medium[91].

IL-17A AND MSCS: THE OSTEOGENIC LINK

Bone homeostasis is a finely regulated process relying on the interplay between the immune and musculoskeletal systems[92]. Indeed, the skeletal and immune systems share several regulatory biomolecules, including growth factors, proinflammatory and inflammatory cytokines, and other signaling molecules[93]. Inflammation plays a strategic role in bone homeostasis and turnover in several inflammation-associated diseases and events, such as bone fracture healing, periodontitis, erosive arthritis, osteoarthritis (OA), chronic rhinosinusitis (CRS), and spondyloarthropathy[92,94].

Several immune cell types (*e.g.*, macrophages, neutrophils, and T cells) infiltrate injured bone tissue and regulate new bone formation during normal and dysfunctional bone repair and regeneration. In this sense, cytokines such as IL-6, TNF-α, and IL-17A positively contribute to the healing process. However, the same cytokines can enhance inflammation, triggering dysfunctional bone tissue regeneration and bone-associated inflammatory diseases[92]. Thereby, the immune system interacts closely with the bone system in health and disease.

By regulating bone regeneration and homeostasis, IL-17 also acts on MSCs' differentiation ability. MSCs express high levels of IL-17RA as well as the other four members of the IL-17R family[95,96], and IL-17A can induce MSC proliferation, migration, and differentiation[97]. For example, in mouse BM-MSCs, IL-17 increases CFU-F (colony-forming unit fibroblasts) average frequency and colony size and cell proliferation, mediated by p38 and ERK1,2 MAPKs[98,99]. Moreover, IL-17A induces the motility and transendothelial migration of peripheral blood MSCs *in vitro*, suggesting a possible role for IL-17 in the mobilization and recruitment of MSCs to injured tissues [95,100]. Consistently, IL-17A also induces the gene expression of matrix metalloproteases-1 and -13 in MSCs, which potentiates their capacity to degrade collagen and invade the ECM[101].

Moreover, IL-17 promotes the immunosuppressive function of mouse BM-MSCs by inducing nitric oxide (NO) and programmed death-ligand-1[102]. In addition, it enhances human BM-MSC-induced inhibition of T cells, and IL-17A-treated MSCs promote regulatory T cells expansion and function, further increasing their immunosuppressive effect[103,104].

THE PRO-OSTEOGENIC ROLE OF IL-17

One of the first pieces of evidence that IL-17 may regulate MSC osteogenic differentiation was provided by Huang *et al*[97]. Primary human MSCs under IL-17 treatment responded with increased proliferation and migration alongside activation of the TRAF6-ACT1-NADPH oxidase (NOX)1/reactive oxygen species-MEK-ERK MAPK pathway axis. Furthermore, IL-17 treatment induced ALP expression and activity with subsequent mineralization in cell culture. Moreover, IL-17 induced osteoclastogenesis of mononuclear cells in coculture conditions with primary human MSCs by induction of macrophage colony-stimulating factor and RANKL in primary human MSCs. Thus, IL-17 contributes to bone turnover by modulating osteogenesis and osteoclastogenesis [97].

The effect of IL-17 on MSCs' osteoblastogenesis can depend on their inflammatory stage or polarization. MSCs display two polarized phenotypes based on the expression of the surface marker Toll-like receptor (TLR): $TLR4^+$ MSCs (also called MSC1) and $TLR3^+$ MSCs (also called MSC2), with different inflammatory functions[105]. *In vitro*, IL-17 induces MSC2 polarization in mouse-derived MSCs through the WNT10b/Runx2 axis, concomitant with increased mineralization rates. Furthermore, in a mouse model of ankylosing spondylitis (AS), MSC2 polarization was related to new bone formation, and the PBMCs of AS patients with new bone formation expressed significantly higher IL-17A mRNA levels than those of healthy donors[106].

Interestingly, osteocytes may enhance the capacity of IL-17 to induce osteogenic differentiation of murine BM-MSCs. IL-17 triggers osteoblastic differentiation *via* the activation of AKT, STAT3, and ERK1,2 along with ALP, Runx2, OCN, and COL-1 expression. The coculture of osteocytes with MSCs under IL-17 treatment leads to an increase in IL-6 and IL-1β secretion by both cell types, which mediates the enhanced osteogenic differentiation of MSCs. Blocking either IL-6 or IL-1β inhibits IL-17-mediated activation of AKT, STAT3, and ERK1/2 in MSC. Therefore, IL-17 may potentiate MSC osteoblastic differentiation within the bone niche by increasing MSC-osteocyte interaction, further contributing to osteoblastogenesis[107].

IL-17A has recently emerged as a mediator of extensive inflammation and abnormal bone formation in AS, leading to bony ankylosis. Basal levels of IL-17A in bodily fluids (patient serum and synovial fluid) are elevated in patients with AS. Moreover, IL-17 enhanced ALP activity and mineralization in AS-derived MSC-like primary bone-derived cells by activating JAK2/STAT3-mediated both Runx2 and C/EBPβ expression[108,109]. Furthermore, in a biomimetic human periosteum-derived cell (hPDC) model, IL-17 induced osteoblastic differentiation. At the same time, blockage of IL-17 with the humanized monoclonal antibody bimekizumab suppressed serum-induced hPDC osteocommitment in AS patients, as evidenced by Runx2 expression inhibition [110].

IL-17 also plays a role in CRS neo-osteogenesis, a heterogeneous and multifactorial disorder of the paranasal sinus mucosa, which involves bone neo-osteogenesis, especially in recalcitrant CRS patients[111]. Levels of Runx2 and IL-17 were increased in tissue sections from CRS patients with neo-osteogenesis. Furthermore, IL-17A-neutralizing antibodies supported the notion that IL-17 mediates Runx2 expression in mouse mesenchymal precursor C2C12 cells treated with nasal tissue extracts. Thus, these data indicate that Runx2, induced by IL-17A, contributes to new bone formation in CRS patients through its effect on osteoblasts' activity[112].

Furthermore, Ono *et al*[113] showed that γδT cells promote bone formation by producing IL-17A and facilitate bone fracture healing in a drill-hole injured femur mouse model. Here, IL-17A was induced in the early phase of bone fracture healing and seems to accelerate bone formation by stimulating the proliferation and osteoblastic differentiation of mesenchymal progenitor cells. Conversely, bone repair impairment in *Il17a*$^{-/-}$ mice occurs due to decreased osteoblastic-dependent bone formation, while osteoclastic bone resorption is not affected[113]. Furthermore, IL-17 enhances *in vitro* BMP-2-induced osteoblastogenesis in injury-associated MSCs.

Similarly, IL-17 synergizes with BMP-2 to induce osteoclastogenesis in human MSCs *in vitro* and *in vivo*. IL-17 dramatically increased matrix mineralization mediated by BMP-2 in human MSCs[114]. In a rabbit model, IL-17 enhanced BMP-2-induced ectopic bone formation in ceramic scaffolds coated with bisphosphonate zoledronic acid (ZOL) by suppression of osteoclasts. Doubled bone volume was observed after 12 wk of BMP-2 and IL-17 co-delivery compared to only BMP-2 in subcutaneous ceramic scaffold implantation. IL-17 induces connective tissue ingrowth and restores BMP-2-induced vascularization and connective tissue formation inhibited by the ZOL coating [115].

Dental-derived MSCs, which represent an ideal source for tissue engineering, and regenerative and dental medicine[116], also differentiate toward osteoblasts under IL-17A stimuli. For instance, IL-17 induces the osteogenic-associated proteins Runx2, OC, and ALP and mineralization in MSCs derived from dental pulp[117]. Similarly, IL-17A induces *in vitro* osteogenic differentiation in MSCs from human exfoliated deciduous teeth (SHED). IL-17 increases cell proliferation in five days of treatment while inducing ALP expression on day 14 of cultivation. Moreover, stem cell marker c-Myc and Nanog expression were downregulated after IL-17 treatment. This stem cell marker inhibition occurred concomitantly with the upregulation of osteogenesis-associated proteins—such as Runx2, COL1, OPN, OCN, and OPG—along with RANKL downregulation, which increased the OPG/RANKL ratio[118].

IL-17 may regulate RANKL expression in murine primary osteoblasts from the calvaria bone *via* JAK2-STAT3 signaling, which depends on cell autophagy in an IL-17 dose-dependent fashion. Low doses of IL-17 induced autophagy, while high doses activated JAK2-STAT 3 signaling, which could be reversed by autophagy induction with the mTOR inhibitor rapamycin[119]. Conversely, autophagy inhibition by the phosphoinositide-3 kinase (PI3K) inhibitor 3-methyladenine greatly enhanced IL-17-induced JAK2-STAT3 signaling. Furthermore, high IL-17 levels promoted ALP induction and mineralization of osteoblast progenitor cells. This treatment also increased the opg and rankl mRNA transcripts levels, and OPG and RANKL proteins were found along with a decreased OPG/RANKL expression ratio. Thus, IL-17A, depending on the dose, may regulate bone turnover, *i.e.*, osteoblastogenesis/osteoclastogenesis balance, by modulating the OPG/RANKL ratio[119].

Furthermore, IL-17A can interact with and potentiate the osteoblastic function of other inflammatory factors such as TNF-α. When used in combination, IL-17A and TNF-α further enhance ALP activity and matrix mineralization. Moreover, this combination synergistically induced the expression of Schnurri-3, a finger protein that plays a critical regulatory role in skeletal remodeling[120] and inhibits RANKL expression associated with osteogenic induction. Furthermore, IL-17A and TNF-α combination increased the type II TNF receptor (TNFRII), which may explain the synergistic effects on the osteoblastic differentiation of MSCs[121]. Similar effects of

combined IL-17A and TNF-α are observed on fibroblast-like synoviocytes (FLS) isolated from RA and OA patients, albeit with different potency[122].

Meanwhile, in OA, simultaneous bone destruction and osteophyte formation were observed and related to a reduced joint destruction rate[123]. Namely, FLS are cells of mesenchymal origin and are the dominant nonimmune cells in synovial tissues, vital elements in defining the stromal environment within arthritic bone diseases[124]. Both RA- and OA-derived FLS can perform bone mineralization *in vitro* and express Wnt5a under chemical induction, and IL-17A addition further potentiates differentiation. In addition, RA bone explants in *ex vivo* assays showed that IL-17A alone or in combination with TNF-α generates a significant decrease in bone volume over the total volume (BV/TV) ratio, while in OA bone explants, only the combination decreased BV/TV ratio. Besides those specific differences, IL-17A enhances TNF-α-induced osteoblastogenesis in both RA and OA-derived FLS[125].

Interestingly, IL-17A switches the differentiation fate of murine mesenchymal progenitor C2C12 cells. IL-17A strongly promotes osteogenic differentiation in cells cultivated in a myogenic medium mediated by ERK1,2 pathway activation and Runx2 transcriptional activity[112,126]. Moreover, IL-17A strongly inhibits myogenic transcription factor expression and reduces cell migration and urokinase-type plasminogen activator expression[127].

Moreover, IL-17A positively exerts osteogenic induction on murine calvaria progenitor osteoblastic cells under incubation with osteogenic media, since IL-17A further stimulates mineralization, along with mRNA expression of ALP (Alp), OSX (Sp7), bone sialoprotein (Ibsp), and OPN (Spp1). Furthermore, IL-17A significantly enhances healing and bone tissue formation in a mouse calvaria defect model under beta-tricalcium phosphate treatment[128]. Furthermore, IL-17A effectively induces osteogenesis in the spontaneously immortalized murine calvaria pre-osteoblast cell line MC3T3-E1, a widely used model for studying osteoblast biology[129]. For instance, IL-17A, under chemical osteogenic induction, potentiates MC3T3-E1 differentiation towards osteoblastic lineage by activation of PI3K-RAC-β serine/threonine-protein kinase (AKT2). In turn, AKT2 knockdown makes MC3T3 E1 unresponsive to osteogenic induction by IL-17A since Runx2, ALP, OCN, and relative ALP activity and mineralization are almost entirely impaired in these cells[130].

Furthermore, IL-17A synergizes with IL-6 to induce ALP activity on osteogenic differentiation of MC3T3-E1 seeded on hydroxyapatite while increasing the expression of OPG and reducing the expression of RANKL, thus increasing the OPG/RANKL ratio and suggesting the potential to reduce osteoclastogenic response[131]. The main aspects of IL-17A-induced osteogenesis are summarized in Table 1.

Other IL-17 family members also have the potential to regulate MC3T3-E1 osteoblastogenesis. Indeed, IL-17F induced osteogenic differentiation by enhancing ERK1,2/C/EBP-β/Runx2 activity[132]. This observation was confirmed by Croes *et al* [114] in an *in vitro* study where human MSCs increased ALP activity in a dose-dependent response to IL-17.

In addition to osteoblastogenesis promotion, IL-17A conversely affects adipogenesis and chondrogenesis in MSCs. Indeed, IL-17A inhibits the adipogenic differentiation of human MSCs and enhances lipolysis of differentiated adipocytes *via* upregulation of cyclooxygenase-2 expression and a subsequent increase of anti-adipogenic prostaglandin E_2[133]. Noh[134] discovered that IL-17 inhibits human BM-MSC adipogenesis and promotes osteogenesis by upregulating the leptin-JAK/STAT pathway. Also, IL-17A may inhibit adipogenic differentiation of 3T3-L1 cells, a model for adipocyte differentiation, by suppressing pro-adipogenic PPARγ, C/EBPα, and transcription factor Krüppel-like factors (KLF)-15 expression, while enhancing anti-adipogenic KLF2 and KLF3 expression[135].

Moreover, IL-17A inhibits TGF-β3-induced chondrogenic differentiation of human MSCs, mediated by impaired protein kinase A activity with a consequent reduction in SRY-type HMG box9 (SOX9) phosphorylation transcriptional activity. As a consequence, chondrogenesis-associated type II collagen (*COL2A1*), aggrecan (*ACAN*), type X collagen (*COL10A1*), and *ALP* are dose-dependently suppressed by IL-17A[136].

THE ANTI-OSTEOGENIC ROLE OF IL-17A

In contrast to the aforementioned pro-osteogenic function of IL-17A, several studies indicated an anti-osteogenic function of IL-17A (Table 2). IL-17A inhibits proliferation and migration and the osteogenic differentiation of healthy periodontal ligament stem cells through ERK1,2 and JNK MAPK[137]. Similarly, IL-17 suppresses human

Table 1 Interleukin-17 promotes osteogenesis: Summary of the main literature data

Cells	Differentiation effects, differentiation markers, signaling, and transcription factors	Ref.
Primary human BM-MSCs	ALP, mineralization; TRAF6-ACT1-(NOX)1/ROS-MEK-ERK MAPK	Huang *et al*[97]
Mouse-derived BM-MSCs	Mineralization; WNT10b/RUNX2	He *et al*[106]
Mouse-derived BM-MSCs	ALP, RUNX2, OCN, and COL-1; AKT, STAT3, and ERK1/2	Liao *et al*[107]
Human MSC-like primary bone-derived cells	ALP, mineralization; JAK2/STAT3, RUNX2, and C/EBPβ	Jo *et al*[109]
Human biomimetic human periosteum-derived cell	RUNX2	Shah *et al*[110]
Mouse mesenchymal precursor C2C12	ERK1/2; RUNX2	Khalmuratova *et al*[112], and Kocić *et al*[126]
Human BM-MSCs	BMP2 synergy, mineralization	Croes *et al*[114]
Human dental pulp-derived MSCs	ALP and mineralization; RUNX2 and osteocalcin	Yu *et al*[117]
Human exfoliated deciduous teeth-derived MSC	ALP; RUNX2, COL1, OPN, OCN, and OPG	Sebastian *et al*[118]
Primary mouse progenitor osteoblastic cells	OPG and RANKL	Wang *et al*[119]
Human BM-MSC	Enhances TNF-α-induced osteogenesis; ALP and mineralization; Schnurri-3	Osta *et al*[121]
Human fibroblast-like synoviocytes from AR and OA	Enhances TNF-α-induced osteogenesis; RUNX2 and BMP2	Osta *et al*[125]
Murine calvaria progenitor osteoblastic cells	Mineralization; ALP, OSX, bone sialoprotein, and OPN	Kim *et al*[128]
Murine calvaria pre-osteoblast cell line MC3T3-E1	ALP and mineralization; RUNX2 and OCN; PI3K/AKT2	Tan *et al*[130]
Murine calvaria pre-osteoblast cell line MC3T3-E1; Seeded on hydroxyapatite	Synergizes IL-6, ALP; OPG	Sritharan *et al*[131]

BM: Bone marrow; MSC: Mesenchymal stem/stromal cell; ROS: Reactive oxygen species; OA: Osteoarthritis; TNF-α: tumor necrosis factor α; IL: Interleukin; ALP: Alkaline phosphatase.

Table 2 Interleukin-17 inhibits osteogenesis: Summary of the main literature data

Cells	Differentiation effects, differentiation markers, signaling, and transcription factors	Ref.
Human periodontal ligament stem cells	Reduces mineralization and ALP activity, and OC; Activates ERK1/2 and JNK	Đorđević *et al*[137]
Human periodontal ligament stem cells	Reduces ALP activity, and RUNX2, SP7, and OCN expression; Inhibits ERK1/2, p38, and JNK signaling	Jian *et al*[138]
Murine calvaria progenitor osteoblastic cells	Reduces ALP, mineralization, and nodule formation	Kim *et al*[139]
Murine-derived BM MSC	IκB kinase-NF-κB dependent catenin degradation	Chang *et al*[141]
Human bone mesenchymal stem cells	Reduces mineralization; Inhibits RUNX2, ALP, and OPN expression; Wnt inhibition by sFRP1 increased expression	Wang *et al*[142]
Murine calvaria progenitor osteoblastic cells	Reduces mineralization and ALP activity; Inhibits OC expression; Inhibits Wnt signaling by increasing sFRP1 and suppressing sFRP3 expression	Shaw *et al*[143]
Murine calvaria progenitor osteoblastic cells	Increases miR-214 and RANKL expression	Liu *et al*[150]

BM: Bone marrow; MSC: Mesenchymal stem/stromal cell; NF-κB: Nuclear factor-κB; ALP: Alkaline phosphatase; OPN: Osteopontin; OC: Osteocalcin.

periodontal ligament stem cell osteogenic differentiation (by reducing ALP activity, Runx2, SP7, and OCN expression). However, in that case, inhibition of MAPK activation (ERK1,2, p38, and JNK) was involved[138].

IL-17A also inhibits osteogenic differentiation of calvaria osteoblast precursor cells upon chemical induction *in vitro*, as evidenced by reduced ALP expression, mineralization, and nodule formation. Accordingly, IL-17 significantly delayed the *in vivo* filling and repairing calvaria defects[113,139]. Furthermore, NF-κB reduces

osteoblasts' capacity for *in vivo* osteogenic differentiation in a murine periodontal infection model, where IL-17A induced NF-κB transcriptional activity in osteoblasts and osteocytes *in vitro*[140]. Consistent with this, IL-17A inhibits murine MSC osteogenic differentiation *via* IκB kinase (IKK)-NF-κB dependent b-catenin degradation. Moreover, IKK-NF-κB inhibition greatly enhances MSC-mediated bone formation *in vivo*[141]. Consequently, healthy BM-MSCs treated with IL-17 showed impaired osteogenic differentiation when induced with a chemical osteogenic differentiation medium. In addition, IL-17A inhibits Runx2, ALP, and OPN expression and mineralization.

Besides osteogenic inhibition, IL-17 treatment provoked Wnt factor inhibition and increased the Wnt signaling pathway inhibitor sFRP1, a member of the secreted, frizzled-related protein, which mediates IL-17 effects[142]. Similarly, Shaw *et al*[143] demonstrated that IL-17A inhibits calvaria osteoblastic differentiation *in vitro* by inducing sFRP1 and suppressing the expression of sFRP3, a decoy Wnt receptor that may stimulate differentiation through a b-catenin-independent pathway[144]. Interestingly, a study in psoriasis patients indicated that bone loss and low bone formation were correlated with increased serum IL-17A levels. Indeed, two mouse models with chronic IL-17A-mediated skin inflammation showed bone loss and impaired osteoblast activity, whereas keratinocytes, γδT cells, and innate lymphoid cells expressed IL-17A, therefore systemically inhibiting both osteoblast and osteocyte function.

Furthermore, IL-17 treatment *in vivo* and *in vitro* inhibited osteoblast differentiation due to Wnt signaling downregulation, while specific IL-17A blocking antibodies ameliorated bone loss and Wnt signaling[145]. Another potential mechanism linking IL-17A to Wnt signaling reduction could be sclerostin (SOST) upregulation. SOST inhibits the Wnt signaling pathway and bone generation[146]. In coculture conditions, SOST overexpression in adipose-derived MSCs (ADSCs) promoted CD4 T cell differentiation toward Th17 cells expressing IL-17A, concomitantly with ADSCs' impaired osteogenesis and enhanced adipogenic differentiation capacity. Exogenous IL-17A further enhanced ADSCs, overexpressing SOST osteogenic inhibition capacity and promoting adipogenic differentiation[147].

Additionally, IL-17A plays a role in secondary osteoporosis in SLE patients. Human BM-MSCs and SHED transplantation recover bone density and ameliorate structure reduction in MRL/*lpr* mice. The transplantation of human MSCs restores impaired functions and the bone metabolism of recipient mouse BM-MSCs/osteoblasts. The Murine MRL/*lpr* model resembles human SLE disorder, with clinical manifestations due to a Fas^{lpr} mutation that promotes self-reactive lymphocytes' survival[148]. MSCs' effects mainly rely on suppressing abnormal BM IL-17A production in recipient MRL/*lpr* mice, as further confirmed by systemic IL-17A blockage by specific antibodies. The authors suggested two potential mechanisms to explain the MSCs' transplantation effects: MSC integration and differentiation into functional osteoblasts contribute directly to bone regeneration, or proinflammatory cytokines can impair bone regeneration. Therefore, MSCs' anti-inflammatory and immunomodulatory effects may regulate IL-17A production by immune cells at bone defect sites[149].

It has recently been reported that microRNA mir-214 mediates the capacity of IL-17A to inhibit primary murine calvaria osteoblast differentiation *in vitro*[150]. MiR-214 inhibits osteogenesis *in vivo* and *in vitro*[151], and IL-17A increases osteoblast miR-214 production and RANKL expression, promoting osteoclast differentiation in coculture conditions due to the reduction of the OPG/RANKL ratio. Furthermore, knockout miR-214 in osteoblasts decreased *in vivo* osteoclastogenesis. Interestingly, AS patients who manifest bone loss have elevated IL-17A and miR-214 Levels in the serum and synovial fluid, indicating their potential diagnostic value in AS[150].

CONCLUSION

Bone tissue formation and regeneration are highly susceptible to microenvironmental factors that regulate the delicate balance between bone synthesis and resorption. An inflammatory response may influence the proper local cell differentiation after a bone injury to accurately regenerate the tissue. Inflammation precedes bone repair and is crucial for bone healing. As a proinflammatory cytokine, IL-17A is produced at high levels, and its release after bone damage can influence MSCs' fate into early osteoprogenitor/osteoblast cells, which further contributes to bone regeneration and full functional recovery. Despite IL-17's capacity to drive the osteogenic commitment of MSCs, it can also function as an anti-osteogenic factor that causes bone loss. Although

these divergent IL-17A roles in bone formation are still not well understood, various conditions arising from the local microenvironment, the magnitude of inflammation, and the specific nature and stage of osteoprogenitor cells can influence the directionality of IL-17A's function, resulting in specific differentiation outcomes.

From a molecular point of view, it is possible to speculate that two mutually antagonistic signaling pathways in osteogenesis may influence the capacity of IL-17 to function as either a pro-osteogenic or anti-osteogenic factor. In this sense, we hypothesize that, depending on the cell source and culture conditions, the activation of the pro-osteogenic Wnt pathway or the anti-osteogenic NF-κB signaling can regulate the cell decision in response to IL-17[84,152]. Thus, if NF-κB prevails, elevated levels of Wnt inhibitors, sFRPs, and SOTS expression are promoted and may trigger b-catenin degradation, whereby IL-17 is acting as an anti-osteogenic factor. Conversely, if cells exhibit low NF-κB activity, the Wnt pathway can freely operate, and IL-17, in cooperation with this signaling, may function as a pro-osteogenic factor. Accordingly, one potential candidate for controlling NF-κB signaling is IL-10[153], also produced by MSCs[154]. The levels of IL-10 in cell culture may influence NF-κB signaling activity [155] and, thereby, drive IL-17's effect on MSC osteogenic fate. However, this hypothesis needs to be experimentally confirmed.

Moreover, the dual roles of IL-17A might result from species-specific characteristics of MSCs and MSC-derived osteoblasts due to the interplay of various microenvironmental issues that condition IL-17A's effects or mode of action at the cellular level. Although it is clear that IL-17A profoundly affects osteogenic differentiation, further standardized studies are necessary to determine how osteogenic differentiation is either positively or negatively regulated and when IL-17 acts as a pro-osteogenic or anti-osteogenic cytokine. Finally, a deep understanding of the precise inflammatory and tissue conditions may help design better therapeutic strategies for IL-17A-associated bone diseases.

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REVIEW

Why stem/progenitor cells lose their regenerative potential

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Abstract

Nowadays, it is clear that adult stem cells, also called as tissue stem cells, play a central role to repair and maintain the tissue in which they reside by their self-renewal ability and capacity of differentiating into distinct and specialized cells. As stem cells age, their renewal ability declines and their capacity to maintain organ homeostasis and regeneration is impaired. From a molecular perspective, these changes in stem cells properties can be due to several types of cell intrinsic injury and DNA aberrant alteration (*i.e* epigenomic profile) as well as changes in the tissue microenviroment, both into the niche and by systemic circulating factors. Strikingly, it has been suggested that aging-induced deterioration of stem cell functions may play a key role in the pathophysiology of the various aging-associated disorders. Therefore, understanding how resident stem cell age and affects near and distant tissues is fundamental. Here, we examine the current knowledge about aging mechanisms in several kinds of adult stem cells under physiological and pathological conditions and the principal aging-related changes in number, function and phenotype that determine the loss of tissue renewal properties. Furthermore, we examine the possible cell rejuvenation strategies. Stem cell rejuvenation may reverse the aging phenotype and the discovery of effective methods for inducing and differentiating pluripotent stem cells for cell replacement therapies could open up new possibilities for treating age-related diseases.

Key Words: Stem cells; Aging; Self-renewal; Rejuvenation; Aging-associated disorders; Epigenetic changes; Aging environment

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Core Tip: Aging influences the ability of stem cell renewal, inducing a gradual functional decline of adult tissue-specific stem cells in maintaining homeostasis of the tissue and playing a role in the pathophysiology of various aging-associated disorders. Stem cell rejuvenation strategies may reverse this aging phenotype.

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INTRODUCTION

During regular physiology or in response to a damage, many tissues expand and regenerate thanks to resident stem cells. Adult stem cells are unusual in that they can self-renew and differentiate into a number of cell types within a tissue. Stem cells were thought to be immortal because they do not undergo replicative senescence, but it is now known that they are vulnerable to damage accumulation. Because of their location at the top of the hierarchy of cellular lineages, their dysfunction may have a greater impact on the of their progeny and they could fail in tissue recovery.

There are two key hypotheses for the etiology of aging. The first is the theory of "antagonistic pleiotropy", which claims that genes that cause aging are chosen because they offer a gain timely in life[1]. The other is the 'disposable soma' hypothesis, which argues that somatic maintenance is expensive and can only be used as a method to prevent development and reproduction[2].

Predation-prone animals spend extensively in growth and reproduction at the expense of longevity as a result. Many of the processes that promote stem cell aging occur since they provide health and survival advantages during growth or youth, but they are harmful later in life, according to these ideas[3].

Understanding stem cell aging is likely to be important if we consider remarkable regenerative capacity of several tissues such as aging at the organ level of tissues that regenerate continuously. Most mammalian cells undergo a limited number of cell divisions in culture also known as the Hayflick limit[4]. The number of cell divisions allowed in cell culture varies from cell type and species, and only a few types of cells are able to extend this limit.

Embryonic stem cells (ESCs) are unique among all stem cell populations for their virtually infinite capacity to self-renew and pluripotency during embryogenesis. These properties are transferable, and these cells can also reprogram somatic nuclei and presumably confer immortality through somatic cell nuclear transfer (nuclear transfer-embryonic stem)[5]. ESCs protect themselves from senescence through adaptive mechanisms aimed at maintaining a high genetic stability by efficiently repairing DNA damage and maintenance of epigenetic status[6-10]. They have an intrinsic vigorous barrier to aging and can produce soluble pro-regenerative proteins for rejuvenating processes[11]. It is increasingly evident that adult stem cells (also named tissue stem cells) are rests of embryonic growth, and several of the primary developmental pathways are still active or functional in these cell populations to maintain postnatal organ homeostasis and regeneration.

In the plethora of stem cell classification, mesenchymal stem cells (MSCs) also known as mesenchymal stromal cells can be encountered as less potent stem cells, with more distinct capacity of differentiation. MSCs are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes in a way that is dependent from exposure to the particular soluble factors in their microenvironment [12].

The most common and longest utilized adult source tissues for human MSCs are bone marrow and the adipose tissue stromal vascular fraction, thereby these sources provided the majority of literature data in this field. In the last decade, the possibility to activate and mobilize these cells into site of injury (*i.e.* for muscle, heart) led researchers and clinicians to optimize their therapeutic use. MSCs treatment has demonstrated to reduce fibrosis, to stimulate of neovascularization, to promote an

immunomodulation, and stimulation of endogenous tissue regeneration[13,14].

Compared to MSCs, adult stem cells are cells that reside in specialized niches that help regulate stem cell self-renewal and differentiation. They maintain the ability to differentiate into organ-specific cell types and play a role in regeneration and homeostasis of nearly all tissues during life. Adult stem cell functionality declines with age, and different type of cellular injury as well as changes in the niche and circulating blood factors contribute to this age-related decline[15].

Here, we analyze what is known about aging in several kinds of adult stem cells and consider what changes in stem cell number and function are known to occur with aging, what aspects of stem cell performance make them vulnerable or resilient to aging, and how much stem cell function decline leads to aging. Finally, we examine possible cell rejuvenation strategies.

INFLUENCE OF AGING ON THE REGENERATIVE POTENTIAL

Aging is a phenomenon characterized by the time-dependent functional decline that influences organisms[16]. It is identified by nine hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, altered intercellular communication, and stem cell exhaustion[16].

Aging influences the renewal of stem cells and induces a gradual functional decline of adult tissue-specific stem cells to maintain homeostasis of the tissue in which they reside. Thus, aging-induced deterioration of stem cell functions may play a crucial role in the pathophysiology of the various aging-associated disorders[15].

Some studies have shown that the regenerative potential of MSCs is downregulated with age, which limits their therapeutic use[17]. In fact, MSCs coming from aged donors (> 60 years) displayed an increase in senescent markers compared to cells of young donors, and also reduced proliferation ability and differentiation potential[17].

Senescence also affects the regenerative capacity of human adipose-derived mesenchymal stem cells (hASCs) that play an important role in the treatment of degenerative diseases[18]; hASCs are abundant and easy to obtain from patients during surgery[18,19]. Furthermore, the use of these cells is safe and efficient for regenerative medicine[19]. Aged hASCs have a decreased rate of proliferation and chondrogenic and osteogenic capacity with increased senescence relative to younger cells[18].

The effect of aging is also known on multipotent postnatal stem cells isolated from human periodontal ligaments (PDLs) that are used in periodontal regenerative therapies for reconstruction of tissues destroyed by periodontal diseases[20,21]. Periodontal diseases increase with age, which compromise PDL stem cells (PDLSCs). Studies in aged donors have shown a decreased number of PDLSCs and a reduction in osteogenic and adipogenic activity together with a weakening of the differentiation marker RUNX2[20,21].

Several research groups have studied the impact of aging on bone marrow mesenchymal stem cells (BMSCs), which are essential for promoting hematopoietic cells in addition to contributing to bone formation. The aging of BMSCs and/or their response to age-related changes in environmental stressors, such as the extracellular matrix and circulating metabolites, may prolong aging or age-related pathologies. The results of natural chronological aging of BMSCs are yet unknown, although it appears that with chronological age, BMSCs decrease in frequency and progenitor cell capabilities such as proliferation and differentiation reduce. These functions, however, require more investigation in *in vitro* and *in vivo* contexts[22]. Experiments on aged mice show that muscle-derived stem progenitor cells (MDSPCs) have reduced regenerative functions[22,23]. Proliferation and multilineage differentiation are both poor in MDSPCs from elderly and progeroid mice. MDSPCs isolated from young wild-type mice and administered intraperitoneally to progeroid mice, extend their lifespan and improve their health. Moreover, MDSPCs alleviate degenerative alterations and vascularization in areas where donor cells are not detectable, implying that their therapeutic action is mediated *via* secreted factors[23].

Importantly, age has been demonstrated to promote the decline of hematopoietic system by multiple molecular and cell-intrinsic mechanisms extensively reviewed by de Haan *et al*[24].

Hematopoietic stem cells (HSCs) deriving from aging mice and transplanted in younger mice showed lower self-renewal capacity, demonstrating that they are vulnerable to age-related stress and consequently lose self-renewal capacity. This

process is influenced by cell-intrinsic and extrinsic factors and can compromise the immune system. Studying the aging process of HSC is important to develop strategies to improve the quality of life in the elderly, as it can make us better understand the mechanisms of age-related immune diseases[24,25]. Aging is responsible also for the progressive neural stem cells (NSCs) loss of function. Studies on adult mice showed that they have a role in maintaining cognitive functions; aging induces the loss of NSC neurogenesis capacity, with consequent brain degeneration. Biological aging of the brain occurs in several neurodegenerative diseases, such as Alzheimer's and Parkinson's, with dysfunction in the NSC compartments[26,27].

Moreover, numerous studies report an active role of Adult Renal Progenitor Cells (ARPCs) in renal repair processes during acute or chronic injuries. It has recently been shown that tubular but not glomerular ARPCs have a regenerative effect on cisplatin-damaged proximal renal tubular cells preventing apoptosis and increasing the proliferation of surviving cells particularly through their secretome and the TLR2 engagement revealing a relevant functional role of this receptor in directing the repair by renal progenitors[28]. Additionally, ARPCs play an important role in the prevention of endothelial dysfunction and may be employed in new strategies to protect the endothelial compartment and promote kidney repair[29]. Furthermore, recent studies demonstrated that ARPCs can regulate the immune response by inducing Treg cells of the immune system and modulating double negative T-cells, which are involved in the balance between immune tolerance and autoimmunity[30]. All these regenerative properties of ARPCs can be affected *via* renal senescence, which can affect renal progenitors by both causing renal aging and the inability to repair renal damages.

In fact, increased renal expression of cyclin p16INK4a in the tubular epithelium occurs during aging (and to a lesser extent in glomerular (podocytes and parietal epithelium) and interstitial cells). Alterations in p16INK4a were more noticeable in the cortex compared to the medulla[31-33]. In rodents, the quantity of senescent cells in proximal tubules, but not in the glomeruli, increases with age. Moreover, renal tubular cell senescence correlates with tubular atrophy, interstitial fibrosis, and glomerulosclerosis[31,33]. Instead, the removal of senescent tubular cells leads to decreased glomerulosclerosis[34].

Prolonged or repeated renal injury leads to maladaptive repair leading to chronic kidney disease[35]. A possible explanation lies in the accumulation of senescent cells during aging and post-injury because the senescent cell burden slowly accumulates over time after acute kidney injury (AKI)[35]. Additionally, the level of senescence in graft biopsy before kidney transplantation could predict the outcome in terms of graft function[36] suggesting that targeting senescent cells could be an effective therapeutic intervention in kidney disease.

CD133 is a functional and constitutional marker of renal stem cells. In this context, CD133 expression is fundamental in the regulation of cellular senescence[37]. Indeed, CD133 is implicated in Wnt/b-catenin signaling, and its expression limits cellular senescence. CD133 can act as a permissive factor for Wnt/beta-catenin signaling (plays a role in protecting b-catenin from degradation) and plays a role in tissue repair. Furthermore, its absence altered cell proliferation after injury favoring senescence[38].

Bussolati's group demonstrated the role of CD133 expressed by tubular cells during injury and its role in the repairing process. Furthermore, CD133 favored cell proliferation in the regenerative phase and limited cell senescence. In fact, the CD133 expression in ARPCs was reduced by cisplatin, but its expression was re-acquired one week after the cisplatin damage. Instead, CD133-knockdown ARPCs (CD133-Kd) displayed a significantly lower proliferative ability during the recovery phase compared to the normal CD133 ARPCs. Furthermore, the expression of the b-galactosidase senescence marker was significantly higher in the CD133-Kd cells compared to normal ARPCs demonstrating the role of CD133 in preventing senescence [38]. Therefore, aging clearly influences the regenerative ability of ARPCs.

HOW STEM CELLS AGE

Stem cell exhaustion is the result of multiple types of aging-associated damages and it is one of the phenomena responsible for tissue and organismal aging[16]. Many mammalian tissue-resident stem cells display a substantial decline in replicative function as they mature. The renewal ability of human tissues declines with aging of stem cells altering their capacity to differentiate in different types of cells[15]. Moreover, age-related loss of self-renewal in stem cells leads to a reduction in stem cell

number[39]. Nevertheless, it may be possible to generate therapeutic approaches to age-related diseases based on interventions to delay, prevent, or even reverse stem cell aging[39].

Understanding how stem cells age may help understanding the normal aging process at the organ level, specifically in tissues with continuous regeneration[3]. These processes are influenced by various cell-intrinsic and cell-extrinsic pathways [40]. Indeed, recent discoveries have revealed a complex interaction among cell-intrinsic, environmental, and systemic signals linked to stem cell function loss during aging[40].

Researchers have worked to understand the main mechanisms with *in vitro* and *in vivo* experiments. The principal causes of stem cells aging are accumulation of toxic metabolites, DNA damage, proteostasis, mitochondrial dysfunction, proliferative exhaustion, extracellular signaling, epigenetic remodeling, and loss of quiescence[40-42]. Many of these aging mechanisms are in common with differentiated cells but stem cell exhaustion, or the quantitative and qualitative loss in stem cell function with time, has a more important impact on tissue aging compared to differentiated cells and has been postulated as one of the aging causes. Adult stem cells perform a critical function in tissue homeostasis by repairing and regenerating tissues throughout life. They maintain practically all tissues and organs, including the forebrain, bone, and muscle, and stem cell exhaustion, defined as a drop in stem cell number and function, is documented in essentially all tissues and organs maintained by adult stem cells. Furthermore, age-related alterations in hematopoietic stem cell HSC differentiation result in fewer adaptive immune cells being produced[43].

In addition, a decline in protein homeostasis or proteostasis occurs in aging cells with consequent accumulation of damaged and misfolded proteins[44]. This is critical especially for the degenerative disease onset. In addition, a reduced capacity of proteostasis can trigger a condition of endoplasmic reticulum stress that contributes to a loss of regenerative potential of aged HSCs[45]. Since HSCs have an age-dependent decrease in nutrient uptake, it is possible that aging of stem cells may be related to nutrient metabolism as well[40].

The extracellular signals and the microenvironment can affect the stem cells senescence. Stem cells reside in specialized microenvironments called niches, which promote their maintenance and regulate their functions[46]. The aging of niche cells and age-dependent alterations in the acellular components of stem cell niches can cause irreversible or detrimental changes in stem cell function[40].

Age causes a decrease in the number of cap cells and hub cells, which act as support cells for germline stem cells (GSCs) in the testes and ovaries, according to studies in Drosophila melanogaster. The disruption of the stem cell niche disrupts BMP signaling, which is required for GSC maintenance, resulting in lower E-cadherin levels and a weakening of the link between GSCs and cap or hub cells. The GSC niche ages as a result of this mechanism. Overexpression of the BMP receptor rescues the age-dependent decline of GSC[47].

AGING ENVIRONMENT

In 1978, Schofield proposed the 'niche' hypothesis to describe the physiologically specialized microenvironment able to maintain the stem cells phenotype and regulate their functions[48-50].

In the recent years, advancements in our comprehension of organ aging revealed that systemic and niche microenvironment, by the release of soluble factors, can deeply influence the stem cells activity in different tissues ranging from hematopoietic, brain, skeletal muscle or hair follicle[51-54] (Figure 1). From seminal studies, more than twenty years ago, emerged that aged muscle successfully regenerates when transplanted in a young host, and from the other side, young muscle displays impaired regeneration when grafted into an aged host[3]. This heterochronic (*i.e* from individuals of different age) tissue transplant studies, revealed that the age of the host animal was a key determinant factor of the regenerative success of the transplant in muscle, since strictly linked to the decline in stem cells reserve function[55].

Several authors postulated that systemic factors could boost tissue regeneration in young animals while inhibiting regeneration in old animals, and that these factors can regulate the main biochemical pathways that control progenitor cell regenerative characteristics. To test this hypothesis, a new experimental model of heterochronic parabiosis was performed by surgical fusion of the circulatory systems of two mice from different ages, allowing the sharing of circulatory system, thus the exposition of

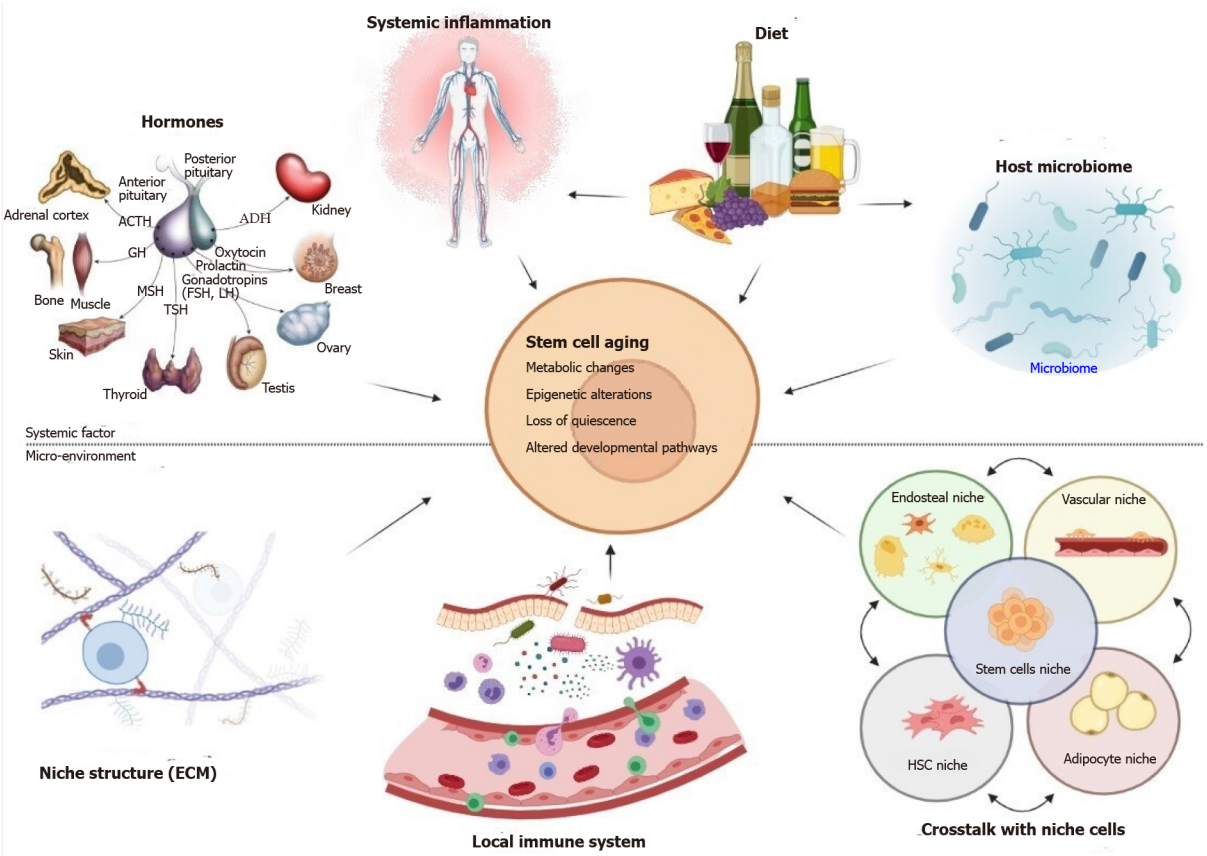

Figure 1 During the aging process, systemic influences and changes in the local microenvironment have an effect on stem cell activity. Changes in systemic signals that control stem cell activity in multiple compartments are caused by a combination of physiological changes in hormone levels, increased inflammation, and the interplay between diet and the host microbiome. Locally, changes in the cellular and molecular composition of the stem cell niche caused by aging cause stem cells to be stimulated abnormally, influencing their quiescence, metabolism, and differentiation ability. Extrinsic signals often cause epigenetic modifications in stem cells, which may result in the aberrant reactivation of developmental pathways. ACTH: Adrenocorticotropin hormone; ADH: Antidiuretic hormone; GH: Growth hormone; MSH: Melanocyte-stimulating hormone; TSH: Thyroid-stimulating hormone; FSH: Follicular-stimulating hormone; LH: Luteinizing hormone; ECM: Extracellular matrix; HSC: Hematopoietic stem cell.

the two animals to the same circulating factors[56].

Parabiosis with young mice (2–3 mo) greatly improved muscle regeneration in the older partner (19–26 mo). Importantly, the activation of resident, aged progenitor cells, rather than the engraftment of circulating progenitor cells from the young partner, was nearly entirely responsible for the improved regeneration of aged muscle. These results indicated that the impaired regenerative potential of aged satellite cells can be improved by means of an increase of positive factors in young mouse serum, a decrease or dilution of inhibitory factors present in old mouse serum, or both. Similar results were also found in the liver from aged mice subjected to heterochronic parabiosis with a young partner. In the context of skeletal muscle stem cells aging (satellite cells), the impairment of Notch signalling leads to diminished regeneration of aged muscle (10). Interestingly, the heterochronic parabiosis restored Notch signalling in aged satellite cells. These findings imply that systemic variables that alter with age can influence the age-related drop in progenitor cell activity. These data have been later confirmed by several groups who performed heterochronic transplantation and parabiosis experiments in several model using aged-satellite cells[3,57,58], neural stem cells, and germline stem cells[3].

It should be noted that these experimental findings could be translated also in clinical settings. In renal transplantation contexts, premature renal aging was found to be modulated by soluble factors. Liu *et al*[59] showed that blood from young mouse was able to reduce acute kidney injury in older mouse, thus a youthful systemic milieu was able to attenuate inflammation, oxidative stress, and apoptosis after renal damage. In addition, transplantation of young bone marrow can rejuvenate the hematopoietic system and preserved cognitive function in old recipient mice[59-62].

NICHE MICROENVIROMENT

The aging microenvironment could be induced by extrinsic inflammatory soluble factors or by a dysbalanced release of intrinsic stem cells protective mediators. In the latter situation, elderly niche cells may be unable to deliver appropriate signals to stem cells, such as morphogen and growth factor signaling, influencing cell destiny decisions (Figure 1).

In mouse muscle's elderly satellite cell niche, the elevated levels of Fgf2 harmfully influence self-renewal[58]. Other circulating factors, such as insulin and IGF-1, that have been correlated to a youthful microenvironment were associated with caloric restriction, as recently demonstrated in growth hormone receptor knockout mice[63].

The extrinsic soluble factors are correlated to the establishment, with the accumulation of senescent cells in aging tissues, of persistent, low-grade inflammatory state called inflammaging, frequently observed in the elderly. Senescent cells secrete inflammatory factors, growth regulators, proteases and other signalling molecules, affecting neighbouring cells in the local environment and promoting senescence and inflammation. The production of a complex mixture of secreted factors is called senescence-associated secretory phenotype (SASP) and includes several cytokines as IL-6, IL-8, CXCXL1, TNF- α, TGF-β, GROa. NF-κB appeared as the central molecular regulator of SASP phenotype. Elevated levels of TGF-β that increase with aging, accumulated in aged muscle of old mice hampering the regeneration and the satellite cell proliferation [64].

Moreover, the pro-fibrotic TGF-β impaired the function of neural stem cells[65], whereas the factor GDF11 showed beneficial effect on the stemness potential of satellite and neuronal stem cells[3].

Taken together, these studies suggest that there are both extrinsic systemic factors and intrinsic niche mediators that can accelerate or delay the aging of stem cells in the niche microenvironment[3,49,50]. A youthful environment can support effective tissue regeneration, whereas an older environment either does not promote or actively hinders it. It will be of great interest to characterize the factors that can modulate the tissue stem cell potential.

It's worth noting that the loss of tissue regeneration potential with age is not irreversible and can be slowed down by controlling systemic variables. These findings show that tissue-specific stem and progenitor cells retain much of their inherent proliferative capability even as they age, but that age-related alterations in the systemic environment and niche in which progenitor cells reside prevent these cells from fully activating for productive tissue regeneration.

MOLECULAR MECHANISMS IMPACTING STEM CELL MARKERS AND PROPERTIES

Aging of adult stem cells is mediated by several molecular mechanisms that are the same involved in the progression of somatic cells aging[41]. This process is the result of multiple mechanisms that act together to induce a progressive decline of stem cell functions, such as regenerative power and in some cases a strong decrease in cell number[41]. Among the principal mechanisms, recent studies reported mitochondrial dysfunction, the release of reactive oxygen species (ROS), DNA damage and telomere shortening, epigenetic modifications and mitochondrial DNA[40,66] (Figure 2).

The decline of stem cell function observed in many tissues during aging is accompanied by complex changes of the chromatin structure including changes in histone modifications and DNA methylation which both affect the transcription of tissue-specific genes[67] (Figure 3).

Mouse embryonic stem cells (mESCs) have higher acetylation and lower methylation levels than differentiated cells, and the chromatin landscape of pluripotent cells has been extensively examined. Increased transcriptional activity and hyperdynamic behavior of chromatin-associated factors in ESCs are consistent with the signatures of a more "active" chromatin conformation.

A co-localization of active and repressive chromatin marks at promoters and enhancers of developmentally regulated genes occurs in mESCs in addition to the surprisingly high dynamics of stem cell chromatin. The H3K4me3 and H3K27me3 chromatin signatures are thought to label genes that are repressed in ESCs but are poised to allow for alternative fates. Mutations in either H3K27 or H3K4 methyltransferase result in severe defects in ESC growth[68].

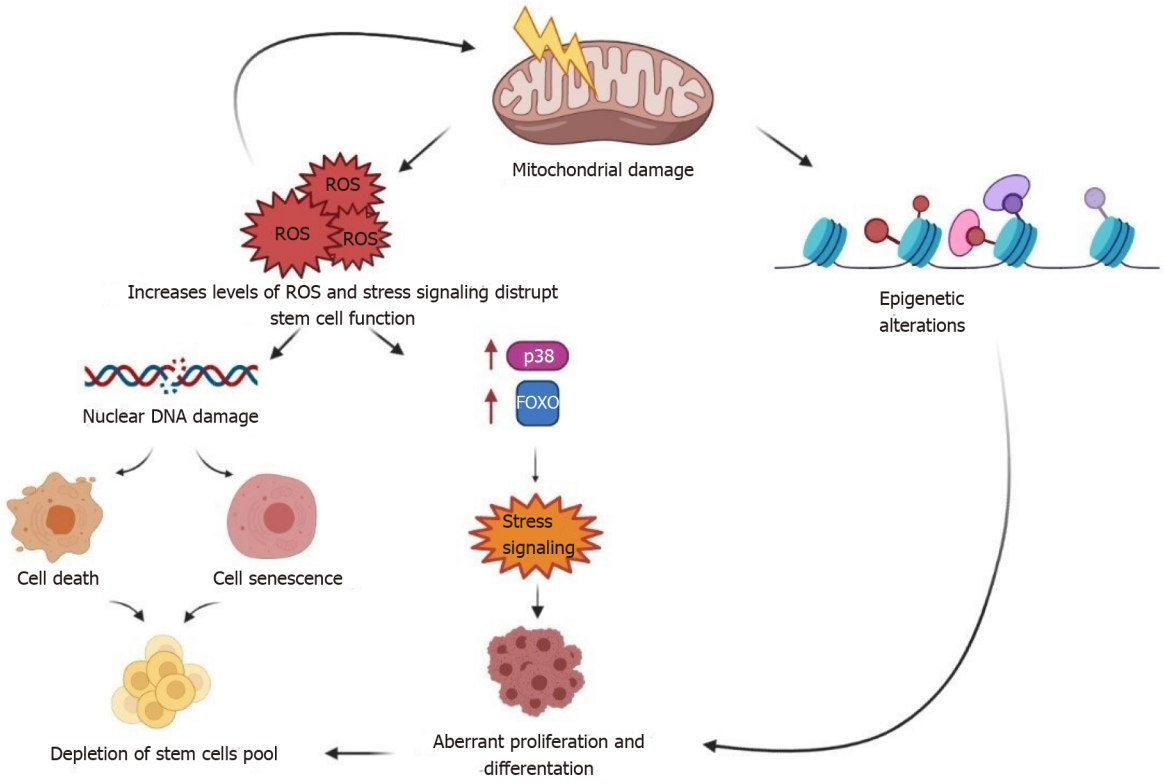

Figure 2 Molecular damage in stem cells as they age. Cell-intrinsic changes in stem cells as they age are intricately linked. In aging cells, mechanisms that would normally ensure the clearance of damaged proteins (autophagy and proteasome-mediated degradation) work badly and cause the accumulation of toxicity and toxic protein aggregates. Excessive reactive oxygen species (ROS) are released by damaged mitochondria, causing further mitochondrial harm (including mitochondrial DNA damage). ROS accumulation causes nuclear DNA damage in aged stem cells, which is aggravated by DNA replication errors and defective DNA repair, leading to cell senescence and apoptosis. Although moderate production of ROS and other stresses are necessary for the regulation of stem cell proliferation and differentiation in normal physiology (at a young age), high levels of ROS trigger stress mediators (p38 and forkhead box protein, resulting in abnormal stem cell function. As a result, stem cell reserves are depleted, and self-renewal is impaired. ROS: Reactive oxygen species.

Current data support the concept that epigenetic regulation erodes in aging stem cells. In *Caenorhabditis elegans*, loss of function of a gene named *Wdr5* extended the life span by about 30% decreasing the levels of a histone methyltransferase that leads to trimethylation of lysine 4 on histone 3. It is unclear why the reduction of H3K4me3 is correlated with longer life span. In contrast, in yeast lower levels of H3K36me3 were found to reduce replicative life span while ablating genes that diminish the mark increased the yeast life span[69,70].

Besides, aged murine HSCs are characterized by an increase in global DNA methylation levels[71]. In line with the findings in *Caenorhabditis elegans*, H3K4me3 Levels tend to rise in aging HSCs particularly on genes involved in maintaining HSC identity. The repressive H3K27me3 mark increased with age also in skeletal muscle stem cells (MuSCs). In particular, this increase was associated with repression of genes that regulate specific differentiation programs in HSCs while it was associated with repression of genes encoding histone genes themselves in MuSCs. Moreover, MSCs from aged individuals have a decline of histone 3 lysine 9 trimethylation—a mark associated with proper maintenance of heterochromatin. However, this is a characteristic of aging of several human adult stem cells[43] (Figure 4).

Another important role in histone methylation of aging stem cells is played by KDM5B. It is a key epigenetic regulator of the H3K4 methylation during cell differentiation, and it acts to reset the epigenetic landscape during differentiation by demethylating H3K4 at the level of self-renewal genes in trophoblast stem cells[72]. H3K4 is implicated in self-renewal activity in HSCs. Moreover, this mark increases with age and covers broader regions.

Studies on epigenetic changes during stem cell aging have been boosted by multi-omic technologies, and these innovative studies in different stem cell types have revealed keys underlying the hypothesis of these age-related epigenetic erosions. Locus-specific alterations in DNA methylation show hypermethylation at promoters of polycomb group target genes and hypomethylation at repeat regions. Analysis of DNA methylome and transcriptome shows an increase in DNA methylation at

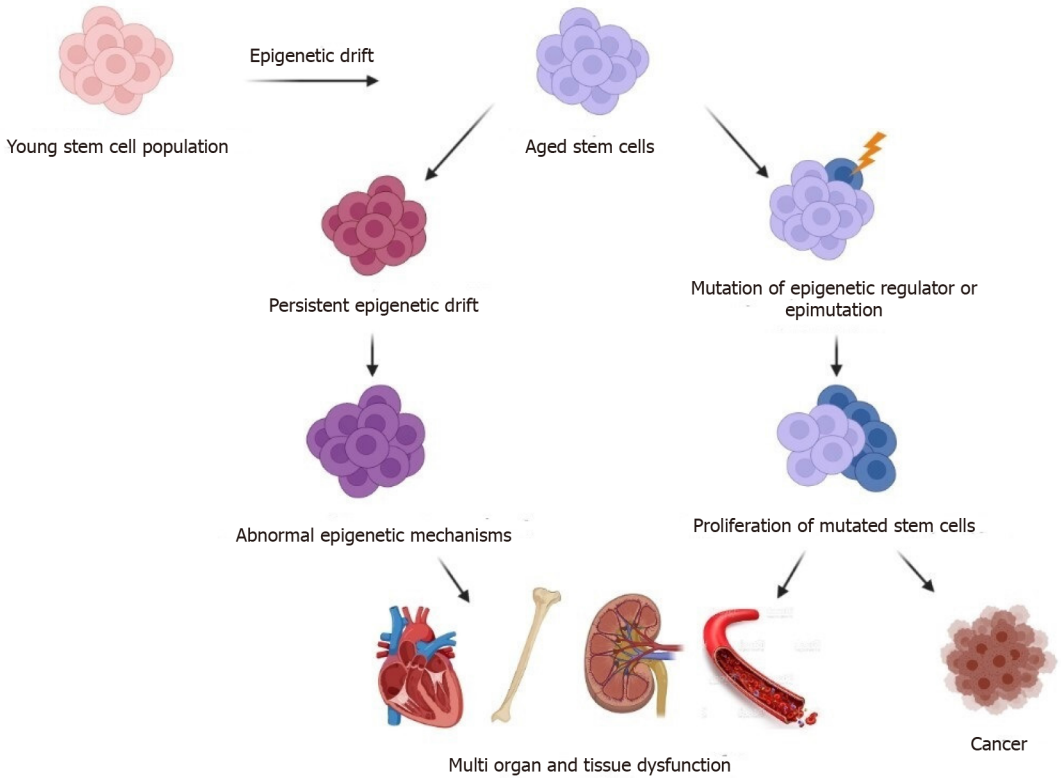

Figure 3 Adult stem cells are altered by epigenetic drift and clonal expansion as they age. The epigenome of adult stem cells undergoes epigenetic drift after reproductive age, which may be caused by molecular disruption, changes in the stem cell niche, or abnormal activation of developmental programs. Depending on the stem cell compartment, DNA methylation and histone modification alterations that accumulate with age can be different and have different effects. Epigenetic drift enhances self-renewal and impairs differentiation of adult stem cells, according to new research. Epigenetic drift causes deregulation of epigenome-sensible gene pathways in certain stem cell compartments, which promotes stem cell dysfunction. Mutations in epigenetic modifiers (for example, DNMT3A), Tet methylcytosine dioxygenase 2, and putative Polycomb group protein ASXL1) have been found frequently in aged haematopoietic stem cells and can lead to the selection of mutant stem cells (blue) that gain clonal dominance, resulting in impairments in adult stem cell differentiation.

promoters of genes associated with differentiation and a reduction at genes associated with HSC maintenance[73].

DNMT1 is the principal DNA methyltransferase in mammalian cells. It is a large and a highly dynamic enzyme with multiple regulatory features that can control DNA methylation in cells[74]. DNMT1 and DNMT3 are essential for SC self-renewal. In human ESCs, deletion of DNMT1 results in rapid cell death[75,76] However, the deletion of DNMT3 promotes HSC self- renewal and impaired differentiation[73,77].

A particular role in the epigenetic regulation of stem cell senescence is played by sirtuin proteins—a class of histone deacetylase enzymes (class III HDACs). SIRT1 plays a role in several stem cell lines, in stem cell differentiation, and regulation of quiescence. This leads to phenotypes typical of aging and premature differentiation [42,78]. In humans, SIRT6-SIRT7 regulated MSC senescence by modulating a hetero-chromatin-LINE1-cGAS-STING axis indicating that condensed heterochromatin is needed to safeguard genomic integrity[79].

A further key-point proteins for aging regulation is Tet methylcytosine dioxygenase 2 (TET2) a member of the ten-eleven translocation enzyme family that converts 5mC to 5hmC and modifies DNA methylation status[80]. TET2 is downregulated in aged NSCs although this can be reversed with parabiosis. Dietary restriction, such as daily or intermittent caloric restriction (CR), affects the transcription of the methylcytosine dioxygenases TET1 and TET3, which are involved in DNA demethylation. Moreover, CR increases the SIRT1-7 enzymatic activity[81]. Some diets could induce epigenetic changes. Interestingly, extra-virgin olive oil can affect histone acetylation processes inducing hyperacetylation of histone H3 in cell cultures[82] (Figure 1).

PIWI proteins can regulate epigenetic mechanisms in some animal models. PIWI proteins play key functions in biological and developmental processes *via* the regulation of cellular mRNAs in addition to their role in transposable element repression[83]. Moreover, they bind small noncoding RNAs called piRNAs (Piwi-associated RNA) and the Piwi-piRNA complex leads to epigenetic regulation[84]. In drosophila, the Piwi are key factors limiting aging-related changes in intestinal stem

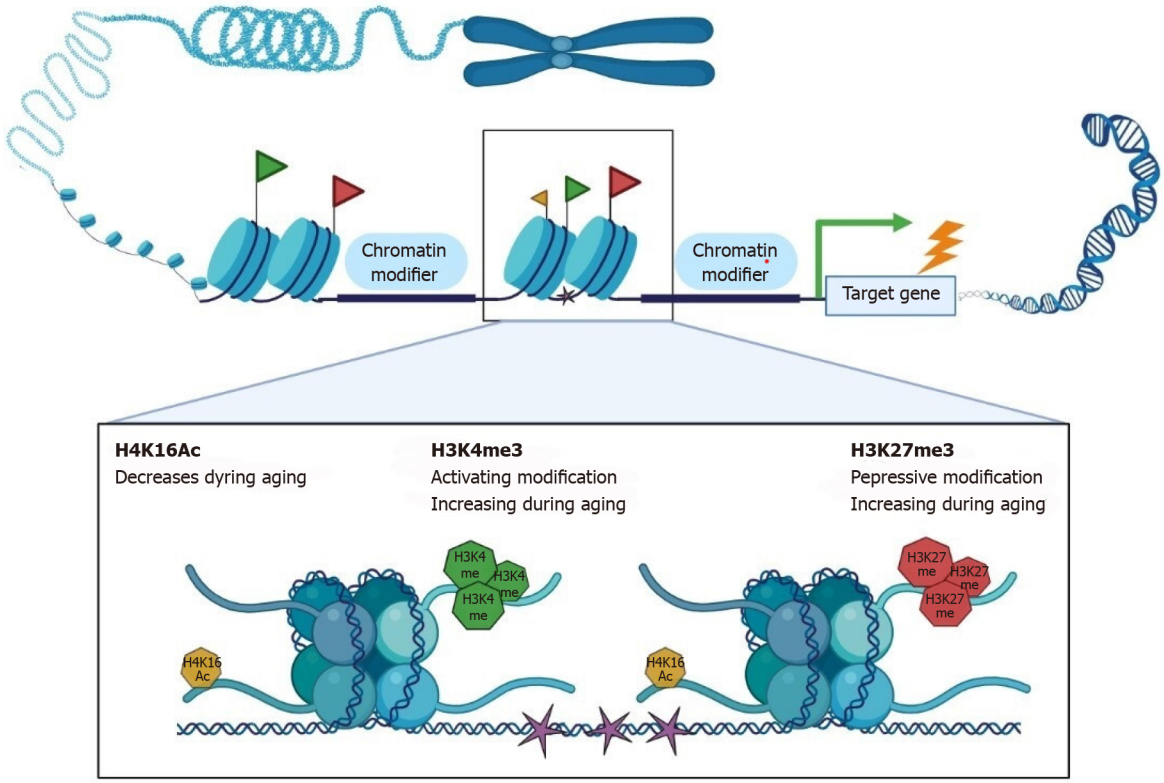

Figure 4 Changes in chromatin related to stem cell aging. In hematopoietic stem cells, satellite cells, and other types of stem cells, changes in the structure and makeup of chromatin during aging have been studied. Differences in chromatin-modifying enzyme levels and distribution (blue oval shapes), histone modifications (green flag, activating; red flag, repressive), and DNA methylation patterns (pink star) are among these shifts. Regional loss of transcriptional silencing altered cell fate decisions, reduced activity, and cellular senescence are all consequences of these changes.

cells[85]. The DNA damage repair system also regulates stem and progenitor cell functions and is affected by aging.

More generally, there is a growing body of evidence that accumulating mutations at stem and progenitor cell level contribute to aging related defects in organ maintenance and lead to cancer development[86].

Therefore, once again, it is clear how a strong connection emerges between epigenetic profiles, genetic elements, and genomic stability in the self-renewal potential of the stem cell. We need to continue to investigate more thoroughly to study sophisticated mechanisms that can regulate these features as well as role that additional methylation/acetylation mechanisms and genetic factors could have in the self-renewal activity and in diseases related to aging. At the same time, it is necessary finalize all of these aspects for the identification of new and more sensible aging markers and therapeutic targets.

In general, stem cells are considered an immortal reserve for tissue regeneration, but several evidences demonstrated that these cells are susceptible to advanced age[66]. Although these cells develop different protective mechanisms to counteract aging-related injury and maintain their self-renew property, their functions started to decline with aging[87,88].

Oxidative stress is still recognized as one of the principal triggers in aging process, determining the impairment of antioxidant pathway and subsequent accumulation of cytoplasmic toxic debris that lead to apoptosis, necrosis or autophagic processes[89]. Within aged tissue, stem cells lose their antioxidant defense mechanisms and can show reduced capacity to regenerate and counteract stress oxidative injury. Several studies found that human MSCs increased ROS levels during progressive replications and became susceptible to oxidative damage activating several pathways and genes involved in aging process such as p53, FOXO1, Nrf2, micro RNAs and long non-coding RNAs[90].

Dysfunctional mitochondria also play a central role in aging process independently of ROS release and accumulation. In DNA Polymerase gamma deficient mice, mutations in mtDNA increased with aging and were correlated to muscle loss and sarcopenia condition[91]. Accordingly, another study demonstrated that mtDNA

mutations in DNA Pol G deficient mice, induced respiratory chain deficiency and premature aging phenotype[92].

In addition, also mtDNA, as nuclear DNA, is exposed to mutations contributing to the development of aging. This process is strongly intensified by the oxidative stress injury and is aggravated by the decline of mtDNA reparative mechanisms in senescent cells[93] (Figure 4).

The mtDNA mutations can be sequenced from induced pluripotent stem cell (iPSC) lines from human skin or blood samples[94]. In this way, it would be easier to analyze and screen mutated genes in mtDNA of iPSC and directly obtaining information of mutated mtDNA of adult cells. Furthermore, it may be possible to find the mtDNA genes involved in several disorders associated with aging and to discover new therapeutic targets[95].

Aging process also alters mitochondrial biogenesis, reducing the number of functional mitochondria and the energy needed for cellular functions. Therefore, the combination of mitochondrial impairment and the decrease of biogenesis leads to aggravation of the aging process[96].

Nuclear DNA damage, induced by several external factors such as radiations, toxins and endogenous mediators like ROS and error in DNA replication mechanism, is associated with accelerated aging. Interestingly, defects in DNA repair processes have been found not just in aging but also in various human progeroid syndromes, which are relatively rare genetic disorders with clinical signs that resemble physiological age, such as hair loss, short stature, skin tightness, cardiovascular disease, and osteoporosis: the Werner syndrome, Bloom syndrome, xeroderma pigmentosum, trichothiodystrophy, Cockayne syndrome, or Seckel syndrome[97]. The role of Nrf2 in cell fate determination and cellular ROS control of HSCs and human airway basal stem cells was later discovered in studies on Keap1-knockout mice[98,99]. Nrf2 is involved in stem cell aging and in HSC homeostasis. Partially through direct association between Nrf2 and CXCR4, Nrf2 deficiency induces cell-intrinsic hyperproliferation and impaired HSC migration and retention in the bone marrow niche[98,99].

Another important key player in aging process is the telomere shortening, widely observed in human and mice studies[90]. In contrast to somatic cells, both embryonic and adult stem cells express telomerase, a reverse transcriptase enzyme (TER), and telomerase RNA component (TERC) which induce the extension of telomeric sequences and reduce the telomere shortening process[90]. TERC provide the template sequence for reverse transcription and help to assemble the ribonucleoprotein complex during maturation process. The interaction between TER and the protein component telomerase reverse transcriptase determines the catalytic activity, processivity, and telomere-binding ability of telomerase[100]. When defective, they can induce premature aging. Several studies showed the importance of telomerase enzyme activity to extend lifespan, reduce aging process[90] and avoid cancer development[86] .Emerging evidences underlined the involvement of several miRNA in stem cells functions such as potency, differentiation and self-renewal[101,102]. In addition, each type of stem cell contains a specific miRNA profile. Interestingly, some miRNA confers to stem cells the capacity to respond to several injury and to prevent the development of aging[101,102]. Thus, miRNA could be used in rejuvenate therapies, in order to counteract several diseases associated to aging, like myocardial infarction, neurodegenerative diseases, blood diseases, and muscle[101,102].

Although several molecules and pathways were widely described in aging process to determine and monitor senescent cells, specific and univocal markers are still missing.

The principal features to identify senescent cells include changes in cellular morphology, increased SA-β-gal activity, alterations in chromatin state, modification of gene expression of important kinases involved in cell cycle (*p16*, *p21*, *p53*), telomere shortening and the acquirement of SASP phenotype[103].

Considering that senescence can modify cellular functions, the percentage of senescent stem cells is evaluated by monitoring stem cell state and functions.

Recent studies discovered new senescence markers that could help to better characterize senescent stem cells. Among them, TRAIL (TNF-related apoptosis-inducing ligand) receptor CD264 that has been proposed as a marker of bone marrow-MSC cellular age and it was significantly associated with increased *p21* expression profile and negatively correlated with proliferation[104].

Also, CD143 was found to be expressed in senescent MSC[105]. Therefore, high-throughput immunophenotypic analysis could be an advantageous method to discover senescent cells and in same time characterize their identity[105]. In addition, the rapid turnover of cytoskeleton filament actin in senescent cells could be studied by real-time labelling with a fluorogenic probe and is strongly associated to aged MSC *in*

vitro system[106].

Together these approaches could be useful to identify senescent adult stem cells and to discover new therapeutic strategies to overcome physiological and pathological aging.

STEM CELL AGING UNDER PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS: DIFFERENCES

Adult stem cells undergo aging process both in physiological and pathological conditions. Senescent MSCs play a key role in many diseases especially in age-associated diseases. In osteoarthritis, a small population of MSCs participate in increasing articular cartilage degradation and bone sclerosis[107]. These cells become dysfunctional and senescent thus enhancing cartilage hypertrophy and osteo-degeneration[107].

Idiopathic pulmonary fibrosis is characterized by an irreversible loss of lung function. Here, lung fibroblasts acquire senescent phenotype and modify the microenvironment thus influencing the MSC behavior to sustain the inflamed microenvironment and influence the surrounding cells[108].

Interestingly, cardiac progenitor cells of patients with cardiovascular diseases expressed higher levels of senescent markers and presented reduced self-renewal, differentiation, and regenerative potential[109]. Therefore, these cells negatively impact cardiac-tissue regeneration.

In addition, neural progenitor cells may play important roles in neuro-degenerative diseases including Alzheimer's Disease and Parkinson's[110]. In primary progressive multiple sclerosis, NSC expressed senescent markers and secreted inflammatory mediators like HMGB-1 that negatively influence the microenvironment impairing maturation of oligodendrocyte progenitor cells. Therefore, senescent NSC could contribute to induce aberrant neural aging in several neurological disorders[111].

Considering these studies, we see that cellular senescence is a beneficial compensatory mechanism to avoid accumulation of damaged cells. This mechanism could induce deleterious consequences in stem cells population in older subjects or in the presence of pathological conditions inducing the loss of regenerative capacity. Therefore, there is an increasing need to find therapeutic strategies to promote senescence in tumor cells on one side and avoid this process in stem cells on the other.

CELL REJUVENATION STRATEGIES

One of the main aims of regenerative medicine is the capacity to rejuvenate tissues. This could occur *via* endogenous stem cells or exogenous replacement cells derived from stem or progenitor cells to restore or rejuvenate tissues[112]. Recent discoveries also established that aging is not "irreversible" implying that aging of cells, tissues, and organisms can be "rejuvenated" rather than merely delayed[113]. Recent developments in our knowledge of tissue regeneration as well as the discovery of effective methods for inducing and differentiating pluripotent stem cells for cell replacement therapies promise to open up new possibilities for treating age-related diseases[112]. Reduced ROS levels can be employed to reverse aging phenotypes produced by uncontrolled accumulation of ROS, allowing aged stem cells to reactivate[40].

Treatment with antioxidants such as N-acetylcystein (NAC) and targeting toxic metabolites can considerably improve survival and tissue repair ability of stem cells [114]. NAC treatment enhances the survival of a distinct population of myogenic stem cells in skeletal muscle both *in vitro* and *in vivo* and restores their quiescence and reconstitution capability[40]. In FoxO-deficient mice, therapy with NAC may improve defects in HSC quiescence, survival, and repopulating ability[40].

Furthermore, increasing the activity of DNA repair mechanisms may help stem cells avoid developing age-related abnormalities. Studies in mice show that late-life reactivation of the telomerase RNA component mTERC can reverse degenerative phenotypes in elderly animals that are genetically weak in telomerase activity (due to inactivation of the telomerase RNA component mTERC)[115]. In contrast, increasing telomerase activity may induce malignance tumor.

There are a limited number of studies on rejuvenation of aged stem cells targeting mitochondrial functions[116]. It has recently been demonstrated that PPAR agonists improve the role of hematopoietic stem cells by enhancing fatty acid oxidation[116].

Targeting sirtuins, AMPK, mTOR, NAD+ metabolism, nuclear receptors (such as PPARs and estrogen-related receptors), transcriptional factors/co-factors (such as PPARGC1, FOXO, NCORs), as well as activators of UPRmt, and mitochondrial fusion/fission or mitophagy, are some of the other strategies for improving mitochondrial functions[116]. These approaches, however, must maintain the balance of stem cell self-renewal, proliferation, and differentiation.

Another important treatment was investigated in aged mice with either recombinant GDF11 or oxytocin that reverse the dysfunction of aged satellite cells and restore vigorous regenerative function in aged mice to show that regeneration in aged mice is reversible suggesting that young blood contains humoral "rejuvenating" factors that can restore regenerative function[117].

As a result, in addition to systemic influences, targeting senescent cells and their secretome in aged tissues may help restore stem cell activity[40]. Clearing senescent cells from progeroid mouse tissues through ablation of p16Ink4a-expressing cells delays the onset of diseases in many aging organs, including the fat, muscle, and eye, was done using an inducible genetic model for senescent cell ablation. Senescent cell clearance at the end of life did not boost age-related pathologies, but it did slow their development.

Furthermore, senolytics, a new class of drugs that selectively kill senescent cells, represent a great potential for improving health span[118]. They could be beneficial in a variety of age-related pathologies, such as sarcopenia and metabolic disorders[119].

Drugs such as rapamycin can be used to rejuvenate aging cardiac stem cells[118] through the inhibition of mTOR—the major downstream component in the PI3K senescence pathway. This action leads cells from a senescent to a quiescent stage[118].

The WNT/β-catenin pathway is another potential target for rejuvenation of hMSCs used in stem cell therapy for cardiac repair[118]. The WNT/β-catenin pathway is related to stem cell renewal and differentiation through regulation of CTNNB1, which plays a crucial function in cardiogenic development. The age is connected with reduction of MSC proliferation and differentiation and WNT/β-catenin signaling. Lithium therapy increases β-catenin availability to boost myogenic differentiation and can revive some functions of MSCs from aged people[118,120].

However, epigenetic rejuvenation has been proposed to be the safest and most successful form of regenerative medicine. It can delay aging and the onset of age-associated decline and diseases to extend health span and lifespan[81,121]. Different methods can induce epigenetic reprogramming. For example, metabolic manipulation like caloric restriction influences DNA methylation and histone modifications. Another method could be plasma exchange to obtain the same effects of heterochronic parabiosis. Here, the circulatory systems of young and old animals are surgically linked allowing immune cells and secreted factors in the blood to swap. It has rejuvenating effects in old animals, reducing age-related dysfunction in a variety of tissues. Finally, pharmaceutical administration and senescent cells ablation can be useful to alter gene expression and reprogramming aged cells to a younger state[81]. Dasatinib, for example, destroys senescent fat cell progenitors while quercetin kills senescent human endothelial cells and mouse bone marrow stem cells. Quercetin is a flavonoid found all over nature and regulates the function of DNMTs, HDACs, and histone methyltransferases. Quercetin acts as a geroprotector by enhancing self-renewal and restoring heterochromatin architecture in aged MSCs[81].

As a consequence, aging phenotypes may be reversed in these rejuvenation procedures, restoring the regenerative activity of stem cells with therapies that are promise for the treatment of a variety of disorders, including sarcopenia, heart failure, acute kidney damage, and neurodegeneration. Even while geroprotective chemicals have been linked to a "younger" chromatin architecture, further research is needed to understand how these longevity-promoting medications interact with epigenetic networks to halt the aging process.

CONCLUSION

During the aging process, stem cells in various tissues develop defects that prevent them from performing critical functions such as restoring tissue damage and preserving tissue homeostasis. A decline in the maintenance of a healthy proteome, metabolic changes, alterations in intrinsic and extrinsic signaling pathways, DNA damage, and epigenetic changes are all examples of these defects.

Understanding how stem cell aging affects distant tissues and overall health span is just the tip of the iceberg. This line of research is important because it lays the

groundwork for stem cell-based treatments to help people live longer lives. Rejuvenating intervention may restore stem cells function and the possibility to use these cells for therapy.

We highlighted several interventions in this review that have shown or may show tremendous promise in increasing the function of aged stem cells in a range of scenarios. Unfortunately, many of these therapies are not acceptable or unlikely to be clinically applicable (such as transgenic partial reprogramming or heterochronic parabiosis). Small chemical, diet-based, and some microenvironment modification techniques, on the other hand, have shown to be more clinically effective. While diet-based treatments have showed some promise in increasing the function of aged stem cells, they have yet to demonstrate the potential to restore lost function in a person who is already old. Many of the present solutions need more research. The effects of most therapy options addressed have not been tested on every stem cell compartment, leaving gaps in our understanding of their systemic impacts. Furthermore, more longitudinal studies are needed to fully understand the effectiveness of these therapies and to investigate any potential detrimental side effects. Most of the research merely observe the animals for a few weeks before sacrificing them for examination. However, it is unknown whether advances in certain stem cell compartments will produce toxicity elsewhere in the body or if they would lead to long-term stem cell depletion, senescence, or malfunction more quickly with most treatments. Furthermore, much research has concentrated on stem cells derived from disease models, which may not be applicable to aging.

While there has been fascinating research into the secretome's regenerative potential, clinical translation of a secretomic strategy will most certainly be hampered by manufacturing issues and batch-to-batch variability, which reduces consistency. Identifying the most potent secreted factors, or the most effective mixture of secreted components, may be more advantageous. To achieve optimal efficacy, these elements can be manufactured individually and blended in specified ratios. Moreover, Centenarian studies may provide unique insights into the relationship between stem cell aging and longevity[122]. Finally, since epigenome changes are theoretically reversible and there is evidence that epigenome reprogramming can improve tissue maintenance, regenerative ability, and health, the idea of epigenetic incorporation of damage signals as a cause of stem cell and organism aging holds new promise for translational approaches.

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REVIEW

Nanofat: A therapeutic paradigm in regenerative medicine

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Abstract

Adipose tissue is a compact and well-organized tissue containing a heterogeneous cellular population of progenitor cells, including mesenchymal stromal cells. Due to its availability and accessibility, adipose tissue is considered a "stem cell depot." Adipose tissue products possess anti-inflammatory, anti-fibrotic, anti-apoptotic, and immunomodulatory effects. Nanofat, being a compact bundle of stem cells with regenerative and tissue remodeling potential, has potential in translational and regenerative medicine. Considering the wide range of applicability of its reconstructive and regenerative potential, the applications of nanofat can be used in various disciplines. Nanofat behaves on the line of adipose tissue-derived mesenchymal stromal cells. At the site of injury, these stromal cells initiate a site-specific reparative response comprised of remodeling of the extracellular matrix, enhanced and sustained angiogenesis, and immune system modulation. These properties of stromal cells provide a platform for the usage of regenerative medicine principles in curbing various diseases. Details about nanofat, including various preparation methods, characterization, delivery methods, evidence on practical applications, and ethical concerns are included in this review. However, appropriate guidelines and preparation protocols for its

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optimal use in a wide range of clinical applications have yet to be standardized.

Key Words: Adipose tissue; Nanofat; Stem cells; Regenerative medicine; Adipose stem cells

Core Tip: Nanofat, being a compact bundle of stem cells with regenerative and tissue remodeling potential, has greater application in translational and regenerative medicine. Nanofat behaves on the line of adipose tissue-derived mesenchymal stromal cells. Considering the reconstructive and regenerative potential, the applications of nanofat can be extrapolated to various medical disciplines such as orthopedics and sports medicine, plastic surgery, and dermatology.

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INTRODUCTION

Regenerative medicine encompasses a wide range of approaches, including the use of biologics, stem cell therapy, tissue engineering, cellular reprogramming, and gene therapy to curb various diseases[1,2]. These approaches gave a new dimension to "translational medicine" where the local milieu of the diseased tissue or organs was modulated into a regenerative environment to aid in the healing process[3,4]. Among various available regenerative approaches, stem cell therapy has gained significance. Due to its abundance, availability, and accessibility, the use of adipose tissue and its by-products has sharply increased among varied medical specialties and researchers [5-7].

Adipose tissue biology is complex due to the presence of a heterogeneous cellular population structured in a compact and well-organized manner[8,9]. With the presence of mesenchymal stromal cells as progenitor cells within the organizational structure (Figure 1), the adipose tissue is considered a "stem cell depot"[10]. Compared to embryonic stem cells, adipose stem cells (ASCs) have several advantages: Accessibility, harvesting potential, extraction by a non-terminal procedure, and fewer ethical controversies[11]. Adipose tissue extracts contain various pockets of growth factors, cytokines, adipokines, and transcriptional factors which altogether form secretomes. These acellular secretomes possess more biological activity than whole adipocytes[12]. The products of adipose tissues include microfat, nanofat, microvascular fragments (MVFs), the stromal vascular fraction (SVF), adipose-derived stem cells (ASCs), secretomes, and exosomes, which are obtained by minimal or more than minimal manipulation as per the US-FDA guidelines[13].

Utilizing the paracrine effects of the ASCs, the progenitors at the site of interest are stimulated to evoke a clinical response. Upon the addition of peptides, specific growth factors, and cytokines to help in the transfer of secretome molecules containing mRNA and signaling factors to the site of action, their regenerative potential is exponentially increased[14,15].

Out of all the adipose tissue-derived products, researchers are more interested in nanofat and stromal vascular fraction in clinical practices and research since their preparation involves concentration techniques which result in an increased quantity of ASCs[16,17]. Nanofat is one of the richest sources of adipose-derived stem cells and other progenitor cells[16,18-20]. The product "nanofat" is highly attractive in terms of compact pockets of stem cells and biological peptides[16]. There is evidence of the regenerative and tissue remodeling potential of nanofat in dermatological disorders such as scars, wrinkles, pigmentation, chronic wounds, small joints, and certain ligament-tendon targets[21]. Hence, nanofat is a potential adipose tissue product in translational and regenerative medicine.

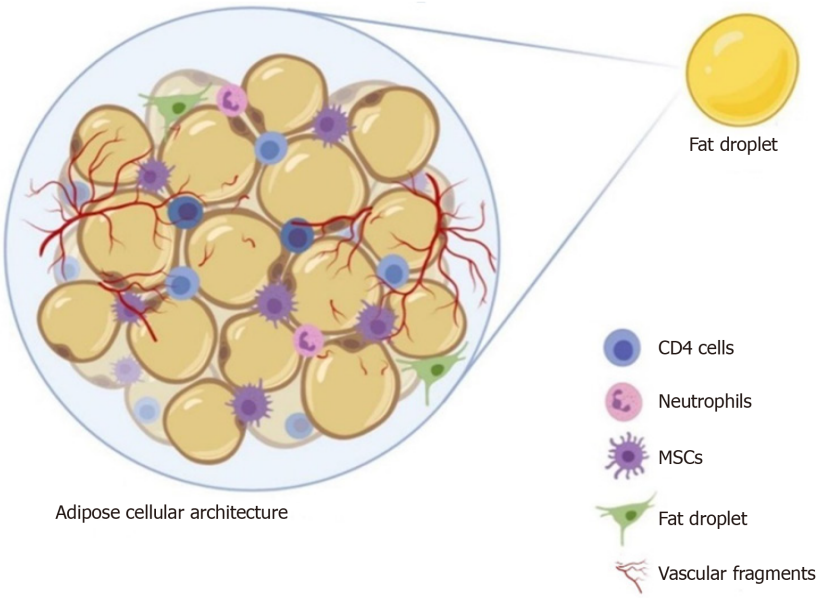

Figure 1 Organisational structure of adipose tissue. MSCs: Mesenchymal stem cells.

NANOFAT

In 2013, Tonnard *et al*[16] developed an injectable byproduct of adipose tissue called "nanofat", which was obtained by emulsification and filtration of the lipoaspirates[22-24]. The mechanical disintegration of adipose tissue is to reduce the particle size and to obtain an injectable product[25]. Such adipose-derived particles called nanofat render stromal cell populations to retain vasculature and naïve cellular matrix[26]. Nanofat is an ultra-purified adipose tissue-derived product that is devoid of mature adipocytes but contains CD34+ rich ASCs, microvascular fragments [fragments of arterioles, venules, and capillaries as they are identified by immunohistochemical staining for CD31 and α-SMA], growth factors [vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), transforming growth factor-beta (TGF-β), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), and granulocyte-macrophage colony-stimulating factor (GM-CSF)], biological peptides [lipoxins, resolvins, protectins, neurotrophic factors, angiogenin, matrix metalloproteinase 9, leukemia inhibitory factor, and macrophage migration inhibitory factor], and cytokines [BMP-2 and -4, IL-1RA, -4, -8, -10, -11, and -13] as shown in Figure 2[16,23]. It is a liquefied, autologous injectable product with the property of biological integration with adjacent cells and tissues due to its homogenous consistency. The size of nanofat components is approximately 400 to 600 μm[27].

Nanofat behaves on the line of adipose tissue-derived mesenchymal stromal cells [28]. At the site of injury, these stromal cells initiate a site-specific reparative response comprised of remodeling of extracellular matrix (ECM), enhanced and sustained angiogenesis, immune system modulation, and cellular turnover[28]. These properties of stromal cells provide a platform for the usage of cellular therapy in various diseases. In 2016, Tamburino *et al* observed the better chances of cellular engraftment of nanofat in various diseases with better functional outcomes[29]. No observation of volume loss, contour irregularities, and liponecrosis was made by grafting nanofat.

ISOLATION OF NANOFAT

In 2013, Tonnard described a preparation protocol (Figure 3) for harvesting nanofat [16]. After the infiltration of modified Klein solution (lidocaine 800 mg/L and adrenaline 1:1000000) in the lower abdomen, adipose tissue harvesting was performed. To obtain "nanofat", the adipose tissue should be harvested with a multiport 3 mm cannula with sharp side holes of 1 mm in diameter. Then the harvested adipose tissue is rinsed with normal saline, followed by filtration through a sterile nylon cloth (0.5-mm pore size) mounted over a sterile canister. Mechanical emulsification of adipose

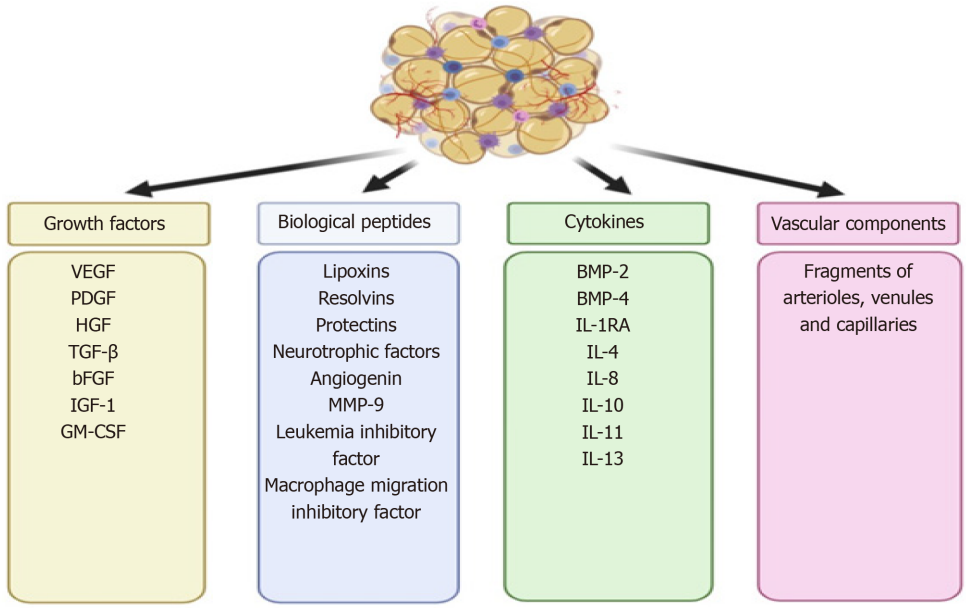

Figure 2 Molecular composition of nanofat. VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; HGF: Hepatocyte growth factor; TGF-β: Transforming growth factor-beta; bFGF: Basic fibroblast growth factor; IGF-1: Insulin-like growth factor-1; GM-CSF: Granulocyte macrophage colony stimulating factor.

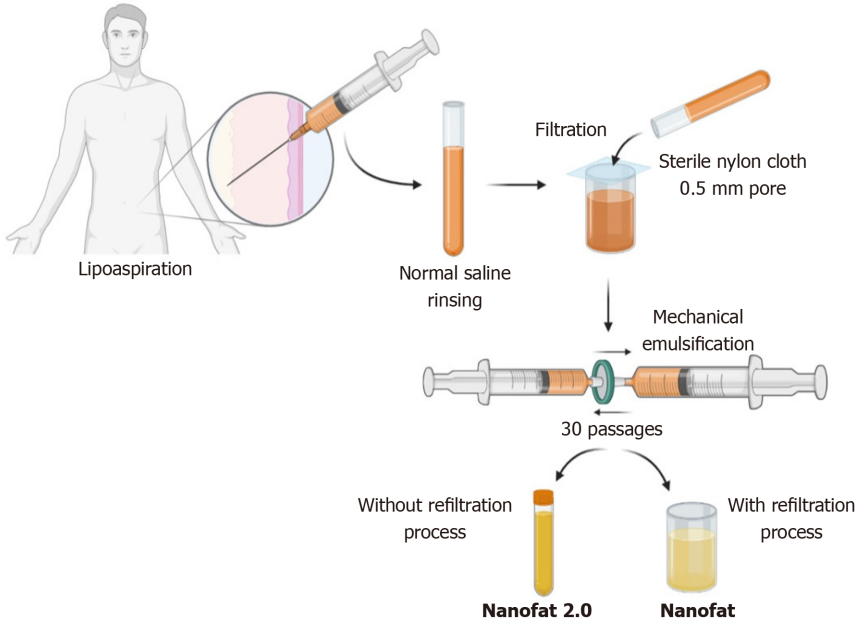

Figure 3 Schematic representation of nanofat preparation.

tissue is done by passing the adipose tissue between two syringes connected by a Luer-Lock connector for a minimum of 30 passages, where the size of the adipose tissue is stepped down with every passage and finally converted into an emulsion. The emulsified adipose tissue appears whitish. The emulsified fatty liquid was again filtered over the sterile nylon cloth and the effluent was collected in a sterile container which is named "nanofat"[16]. Nanofat preparation reduces the processing time, cost, and regulatory constraints compared to the enzymatic digestion of the adipose tissue [30,31].

CHARACTERIZATION OF NANOFAT

Nanofat has gained significant importance in biocellular regenerative medicine[21]. The characterization of nanofat components is based on cellular composition, stromal cell concentrations, and total nuclear counts after membrane lysis. Nanofat is the product of volumetric reduction of mature adipocytes by 95% and contains a concentrated and compact volume of a heterogeneous group of cells equivalent to SVF[21,32]. However, nanofat may be a superior cell source compared to SVF. Sesé *et al*[26] estimated the total cellular load in the mechanically prepared nanofat to 6.63 million cells/g lipoaspirates whereas in enzymatically disintegrated SVF it was 0.68 million cells/g lipoaspirates. The nucleated cellular count in nanofat was 70% and in SVF was 7.3%. The cellular burden in nanofat contains the stromal cellular population which was equivalent to the lipoaspirate[26]. The low yield of cell counts in SVF was shown to be due to inefficient enzymatic digestion and the vast majority of the cells remained within the extracellular matrix[26].

Nanofat provides a higher concentration of bioactive micromolecules at the target or recipient site, which acts as a bridge to enhance the site-secreted chemotactic agents [26]. The cellular components present in nanofat are multi- and pluripotent and have the potential to differentiate into various cell lineages such as adipose tissue or connective tissue. The extracellular matrix present in nanofat likely caters to the cellular components to sustain the survival of progenitor cells in its composition. Hence, nanofat can be extrapolated for pre-clinical and translational research in tissue engineering[33]. Various pre-clinical and clinical experiments have shown that nanofat grafting helped in angiogenesis, immunomodulation, enhancing collagen deposition, and preventing fibrosis[34,35].

The theories of adipocyte survival after fat grafting include the "graft survival theory" (some transplanted viable adipocytes survive and remain alive for a longer period) and "fat regeneration theory" (under ischaemic conditions, adipose-derived progenitor cells get activated and undergo regeneration)[36,37]. Zheng *et al*[38] demonstrated that fat extract co-transplantation with nanofat enhances fat integrity, survival, and activation of more adipocyte precursors with a higher number of CD31 positive blood vessels, and more Ki67 positive proliferating cells. Under ischaemic conditions, nanofat co-transplantation with fat extract demonstrated proangiogenic and anti-apoptotic effects with multi-differentiation potential[38].

CELLULAR AND MOLECULAR CHARACTERISTICS OF NANOFAT

The components of nanofat are the derivatives from adipose tissue; hence, nanofat behaves on the line of adipose tissue-derived mesenchymal stem cells (MSCs) at the cellular and molecular levels. Nanofat contains the lowest number of SVF cells. Hence, nanofat is considered the poorest form of SVF. Mohamed-Ahmed *et al* exhibited higher expression of CD34 and CD49d in ASCs; they also showed that CD34 expression helps in long-term MSC proliferation[39]. ASCs express Runx-1 and ALP after 14 d of passage, which resulted in the extended proliferation, maturation, and differentiation of AD-MSCs. The osteogenic capacity of AD-MSCs was induced by mechanical stimulation of culture along with the addition of vitamin D3, PDGF, and BMP-2[40,41]. ASCs activate adipogenesis by induction of adiponectin, leptin, LPL, perilipin, and fatty acid-binding protein-1 through PPAR-γ and increased lipid vesicle formation compared to BM-MSCs[42]. The decreased potential for ASC-based chondrogenesis was due to the decreased expression of TGF-β-R1 and BMP-2 and -4 [43,44]. The chondrogenic activity of ASCs is identified by the expression of types 2 and 10 collagen, biglycan, aggrecan, and decorin genes in the differentiated cells[45]. ASCs possess a higher potential for adipogenic differentiation than for osteogenic and chondrogenic differentiation when compared with BM-MSCs[39,46,47].

TYPES OF NANOFAT

Nanofat 2.0

The unfiltered adipose tissue was initially called adult staminal cells by Lombardo *et al* [48] since they had higher proliferation capacity than the filtered cells. In 2017, Lo Furno *et al*[49] modified the method described by Tonnard *et al*[16] for nanofat, omitting the final filtration and squeezing the emulsified adipose sample through

nylon cloth. Lo Furno *et al*[49] named this product "nanofat 2.0", which was highly rich in the stromal cell population and possessed an exponential proliferation capacity. Lo Furno *et al*[49] have demonstrated faster epithelization of the wound gap within 8 d by placing nanofat 2.0.

After harvesting the adipose tissue from the abdominal region under low negative pressure through a multiport 3 mm cannula with a hole diameter of 1 mm, the resultant lipoaspirate must be subjected to rinsing, filtration, and mechanical emulsification through serial passages between two 10-cc syringes connected by a Luer Lock connector. The resultant by-product after 30 passages is called "nanofat 2.0" (Figure 3)[49].

Nanofat 2.0 components stained highly positively for CD44, CD90, and CD105, which are the most specific immunohistochemical markers for mesenchymal stromal cells[27,49]. Moreover, they stained weakly positively for CD14, CD34, and CD45, which are the lineage markers for hematopoietic stem cells. Histological studies showed the loss of tissue integrity in nanofat 2.0 but revealed huge numbers of adipose-derived stromal cells and cellular debris[49]. Due to the availability of stromal cells and endothelial progenitor cells, nanofat 2.0 resulted in the healing of wounds and long-standing non-healing ulcers where a large volume of soft tissue augmentation was needed[48]. Lo Furno *et al*[49] demonstrated that nanofat 2.0 possessed increased stromal cell and endothelial precursor density and higher proliferative capacity than nanofat. Since nanofat 2.0 is subjected to less mechanical stress in preparation, the viability of the cellular content of the product could be enhanced compared to nanofat[48-51]. The modified nanofats are described in Table 1.

Vivo nanofat

In 2018, Bi *et al*[52] formulated the preparation of nanofat with a combination of enzymatic disintegration and mechanical emulsification of adipose tissue and named this technique "Vivo nanofat". The harvested lipoaspirate is rinsed with 1 mL of 0.2 mg/mL of collagenase I enzyme and the final volume is incubated at 37 °C for 15 min. The final concentrate is centrifuged at 300 G for 7 min and the supernatant fraction is filtered through a 0.6 mm sized cell strainer. The final effluent obtained is called Vivo nanofat. The cellular viability of adipocytes and stromal stem cells has been preserved to a great extent in Vivo nanofat[52]. Although the authors claim that the concentration of collagenase used (0.075%) was less than the amount used for adipose stromal cell separation, the effects of their concentration in the final derivative need further exploration.

DELIVERY OF NANOFAT

The application and delivery of fat grafting to the recipient site are based on optimal vascularity for adipocyte survival. Nanofat can be delivered through micro-needling, intradermal, subcutaneous, and local infiltration depending on the need of the individual and the disease per se[53-55]. Delivering nanofat through small gauge cannulas reduces the recipient site morbidity, risk of bleeding, and poor graft uptake [56]. In fat grafting, the revascularization starts from the peripheral zones; hence, the center of the graft experiences a longer ischaemic time. Moreover, compared to a single injection, experts resort to repeated doses of fat grafting for enhanced benefits [56]. The fat grafting must be applied to the recipient site by withdrawing the cannula in a "fanning out" pattern.

The size of the cannula is the most important criterion to determine the fat application, graft uptake, and survival in the recipient site. However, there is a lack of consensus among studies on the ideal size to be utilized. While the conventional recommendation is to use a cannula less than 2.5 mm diameter to enhance the vitality of fat graft, but Erdim *et al*[59] did not note similar findings in their study on cell viability with differing needle gauge sizes[57-60].

APPLICATIONS OF NANOFAT

Stem cells are an important component of regenerative medicine with increased significance and use in clinical applications. The newer concept of "Regenerative Surgery" has a great scope in augmenting and managing soft tissue defects and reconstructive procedures[61,62], of which adipose tissue-derived nanofat is gaining rapid attention.

Table 1 Modified nanofats

Type	Total volume of lipoaspirate	Procedure
Supercharged nanofat[24]	L + 80 mL	(1) First 80 mL lipoaspirate – Automatic filtration (120 μm filter) and centrifugation at 1300 rpm for 10 min in a closed system with 20 mL Luer lock syringes. The lower SVF was collected and further filtered to obtain the final 20 mL product; (2) Second 80 mL – Emulsification (30 passages) into two 10 mL Leur lock syringes; and (3) The SVF suspension obtained from the first lipoaspirate fraction should be mixed with the emulsified fat to form a supercharged nanofat
Evo modified nanofat[24]	L	(1) Slow centrifugation at 80 rpm for 3 min; and (2) Homogenization of emulsified fat through Luer lock system (20 passages)
Centrifuged modified nanofat [24]	L	(1) Direct centrifugation at 1300 rpm for 10 min; and (2) After discarding SVF, the concentrated aspirate of centrifuged fat should be emulsified by 30 passages through the Luer lock system

SVF: Stromal vascular fraction.

From the early 20th century, autologous fat grafting has gained much attention in the field of biocellular regenerative medicine and tissue engineering. Autologous fat grafting and the products of adipose tissue fragmentation have been used to restore the volume of soft tissue defects in the field of plastic surgery and soft tissue reconstruction. Considering the regenerative potential of adipose tissue, researchers are exploring to identify the key element responsible for its function. The adipose cells were considered the storehouse of progenitor cells and bioactive micromolecules[30]. By concentrating the progenitor cells within the adipose tissue complex, the regenerative capacity of the adipose-based products is enhanced to aid in their applications[21].

Nanofat grafting enhances neoangiogenesis without producing any visible scars and provides a favorable outcome in aesthetic medicine for breast, buttocks, and genital augmentation, facial rejuvenation, and facial volume augmentation[63-66]. The preclinical and clinical studies with the usage of nanofat have demonstrated the regenerative capacity of nanofat.

Plastic surgery

Autologous fat transplantation or lipofillers remain the most suitable management modality available for breast reconstruction. Adipose tissue-derived nanofat can maintain natural breast shape and conceal the underlying prosthesis while augmenting breast size[67,68]. In gluteal augmentation, fat grafting can replace implant-based gluteal augmentation if the patient has adequate and available fat stores [69,70].

Nanofat injections can reduce the atrophic scars due to the presence of adipose tissue-derived stromal cells and avoid the need for surgical procedures[32]. The underlying mechanisms for scar retraction by nanofat are uncertain. Nanofat components can regenerate dermis and subcutaneous fatty tissues and enhance the dermo-epidermal junction. They regenerate by laying down adipose tissue-derived ECM, collagen deposition, and neoangiogenesis[71,72].

Zhang *et al*[73], in a preclinical study, emphasized the scar reduction in rabbit ears by decreasing the α-SMA and collagen type I gene expression and enhancing collagen deposition with the usage of adipose-derived MSCs. Adipose tissue-derived MSCs restore collagen fibrillary organizations and downregulate the fibrosis of the scar tissue. Klinger *et al*[74] described that autologous fat grafting allows the skin to rejuvenate more softly and flexibly, and matches the color of neighboring skin which could be utilized to rejuvenate the texture and color of the skin of the scars present in joints, eyelids, face, and mouth.

Burns: With the advancements in tissue engineering, it is now possible to regenerate the burnt and scarred tissues with minimal scarring and donor site morbidity. Nanofat grafting beneath and within the substance of the scar improves the quality, integrity, and texture of the scar[75]. The histological evidence of fat grafting to scar demonstrates the hyperplasia of dermis and epidermis, vasculogenesis, and collagen deposition. Clinically, the fat grafted scar shows improved scar tone, texture, thickness, elasticity, flexibility, and color of the scar along with reduced scar size[76,77].

Dermatology and aesthetic surgery

The most common procedure for managing facial aesthetics is autologous fat transplantation. Though the transplanted adipose tissue gets absorbed easily, a few progenitor cells stimulate the process of regeneration. The cells present in nanofat in combination with platelet-rich fibrin (PRF) enhances the proliferation and adipogenic lineage differentiation.

Due to this combination treatment with nanofat and PRF, a trend towards the disappearance of wrinkles and improved facial contour and skin rejuvenation have been observed attributable to the autocrine and paracrine effects of stromal cells in nanofat and anti-aging properties of PRF[44,69,70]. This combination treatment enhances the long-term benefits and is being increasingly utilized in the restoration of facial contouring in the field of aesthetic and cosmetic medicine. The skin texture, elasticity, and moisture, and facial rejuvenation can be achieved with nanofat admixed with PRF[78].

In a pre-clinical trial, nanofat injection improved the thickness of the dermal layer and promoted angiogenesis in the photoaged skin of a nude mouse[79,80]. A wide range of improvements were seen in wrinkles, discolorations, and scars due to burns with nanofat applications[81-83]. Liang *et al*[84] emphasized that the combination treatment of nanofat and PRF improves facial depression when compared with hyaluronate filler.

Aesthetically, nanofat grafting is used for the correction of dark circles[85,86], malar bags[56], hollow eyes[86], and blepharoplasty[87]. Due to fat atrophy in the aging process, nanofat has emerged as a plausible technique for facial rejuvenation[88-90].

Apart from being a primary essential tool in revision rhinoplasty, nanofat is being increasingly used in primary rhinoplasty procedures also[91]. Nanofat is being used to correct slight skin irregularities which do not require cartilage grafting. Moreover, considering the cost of the revision rhinoplasty, nanofat grading is being employed frequently as a cost-effective procedure[92,93].

Orthopedics

Due to the wide range of reconstructive and regenerative potentials of nanofat, the applications of nanofat can be extrapolated to orthopedic surgery. The mechanically emulsified adipose tissue can regenerate the degenerated and diseased tendon, ligaments, and articular cartilage[28].

Segreto *et al*[94] evaluated the role of a combination of nanofat grafting with autologous PRP in non-healing infected wounds. The application of nanofat with the micro-needling technique improved the delivery of cellular components into fibrosclerotic tissues and enhanced the regeneration of soft tissue in chronic non-healing wounds. The addition of autologous PRP along with nanofat enhances the proliferative capacity and motility of adipose tissue-derived stromal cells[95,96].

Due to the multi-differentiation potential of adipose tissue, which is a component in nanofat grafting, it could be extrapolated for utilization in avascular necrosis of the femoral head, mild to moderate grades of osteoarthritis of knees, tendinopathies, and non-union of fractures.

COMPLICATIONS OF NANOFAT GRAFTING

The lesser the fat graft is manipulated and the sooner it is injected, the higher the chances of its survival in the target site[97]. Minor complications related to the harvesting are due to the liposuction technique. The possible complications range from bruising, hematoma formation, donor-site pain, infection, contour irregularities, and damage to the underlying structures when the aspiration cannula enters peritoneal or muscular territories[98-103]. Breast augmentation with lipofilling was associated with complications such as fat necrosis, oil cyst formation, and calcifications when performed in large volumes into poorly vascularized areas. Cellulitis at the donor site [104], transient digital numbness[105], infections at both the recipient and harvest sites [106], and cyst formation[106,107] in 10% of hand rejuvenation patients, along with the common complications of fat grafting such as temporary dysaesthesia[106], fat necrosis[106,108], and reabsorption of the grafted fat[107] were also reported.

Facial rejuvenation by lipofilling involves complications related to the fat graft injections in "dangerous" areas of the face such as the glabella and nasolabial folds[88, 109]. Accidental intra-arterial injections may result in cerebral or ocular artery thrombosis resulting from the reflux of fat into the ophthalmic artery and the internal carotid artery[88,109]. To prevent such devastating complications, confirmation of the

absence of blood reflux into the syringe before injecting the graft is a necessary routine, along with a slow pace of injection at low pressure, and the use of a blunttip cannula [88,109].

ETHICAL CONCERNS WITH NANOFAT GRAFTING

Therapeutic use of cellular products, including human cells, tissues, and tissue-based products, comes under the regulation of the Food and Drug Administration (FDA) in the United States and the European Medicines Agency in the European Union[110-112]. For a cellular product to be approved by the regulatory authorities, it should be minimally manipulated and intended for homologous use. Moreover, the entire procedure must be performed on the same day[112]. The main concern with this clause is to clarify the applications which account for the "homologous" use.

The FDA, while formulating these guidelines regarding fat-based therapeutic products, has considered only the adipocyte, not taking into account the potential constituents of the extracellular matrix such as multipotent stromal cells, pericytes, and endothelial precursor cells and restricted their approved usage only to the spectrum of disorders homologous to the utility of only the adipose lineage cellular component of the tissue complex. Appropriate homologous use of these heterogeneous populations with undesignated cellular capabilities needs to be clarified. Since nanofat is processed in a non-enzymatic method, it comes under the minimal manipulation norms of the FDA guidelines. Moreover, it is possible to procure, process, and place the cells in the target environment in a single surgical procedure, thereby reducing the need for additional procedures and the risk of contamination or genomic instability. The functional properties of extracellular matrix fragments, cellular debris, and blood cells in the heterogeneous composition of nanofat need to be defined. Consequently, problems of reproducibility and standardization methods may arise considering the subjectivity involved in the preparation process[113]. Hence, it is challenging to compare the efficacy of product protocols even when they are used for similar scenarios[114]. Therefore, increased efforts to optimize the preparation protocols with standardized methods of tissue manipulation for clinical purposes and analysis of grafting are needed.

CONCLUSION

Nanofat, being a compact bundle of stem cells with regenerative and tissue remodeling potential, is a potential adipose tissue product in translational and regenerative medicine. Considering its wide reconstructive and regenerative potential, the applications of nanofat can be extrapolated to various disciplines. However, appropriate guidelines and preparation protocols for its optimal use have yet to be standardized for its vast range of clinical applications.

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MINIREVIEWS

Application of adipose-derived stem cells in treating fibrosis

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Abstract

Fibrosis is the hyperactivation of fibroblasts that results in excessive accumulation of extracellular matrix, which is involved in numerous pathological changes and diseases. Adipose-derived stem cells (ASCs) are promising seed cells for regenerative medicine due to their bountiful source, low immunogenicity and lack of ethical issues. Their anti-fibrosis, immunomodulation, angiogenesis and other therapeutic effects have made them suitable for treating fibrosis-related diseases. Here, we review the literature on ASCs treating fibrosis, elaborate and discuss their mechanisms of action, changes in disease environment, ways to enhance therapeutic effects, as well as current preclinical and clinical studies, in order to provide a general picture of ASCs treating fibrotic diseases.

Key Words: Adipose-derived stem cells; Fibrosis; Immunomodulation; Modification; Therapeutic effect; Clinical trials

Core Tip: Fibrosis is involved in various diseases. Adipose-derived stem cells (ASCs) are promising candidates for regenerative medicine and anti-fibrosis treatment. We herein discuss the mechanisms of action, changes in disease environment, ways to enhance therapeutic effects, as well as current preclinical and clinical studies of ASCs treating fibrotic diseases in order to provide a general picture.

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INTRODUCTION

Fibrosis is a common pathological feature in various diseases. It can involve multiple systems and organs, for example, lungs, liver, kidneys, skin, heart and skeletal muscle. Fibrosis is characterized by the hyperactivation of fibroblasts, causing them to transit into contractile myofibroblasts, as well as increased synthesis of extracellular matrix proteins, resulting in excessive deposition of collagen fibers, often in the form of abnormally arranged bundles. Macroscopically, fibrosis leads to the stiffening of tissues, often accompanied by the loss of normal functions[1-4].

Adipose-derived stem cells (ASCs) are a promising candidate for regenerative medicine due to their multifaceted functions, abundance, and lack of ethical problems. Numerous studies have looked into the effectiveness and mechanisms of action of ASCs in treating fibrosis-related diseases, as well as the ways to boost their functions [4-7].

In this review, we summarize and discuss recent studies in the hope of providing a general picture of the present applications of ASCs in fibrotic diseases.

MECHANISMS OF ACTION

Interactions with the TGF-β/Smad axis

The transforming growth factor-β (TGF-β)/Smad axis is one of the critical players in the wound healing cascade, whose dysregulation leads to pathological fibrosis[8]. In addition to directly stimulating the synthesis of collagen and fibronectin, TGF-β1 also inhibits the extracellular matrix (ECM) decomposing enzyme, matrix metalloproteinases (MMPs), as opposed to the anti-fibrotic tissue inhibitor of metalloproteinases (TIMPs)[9]. By promoting the synthesis while inhibiting the degradation of ECM, the activation of the TGF-β/Smad axis results in excessive accumulation of ECM, which is characteristic of fibrosis. Another important feature of fibrosis is the transition from fibroblast to myofibroblast, which is also induced by TGF-β1[10]. Various studies[10-13] have shown that the decreased expression of TGF-β1 is an important effect of ASCs in the treatment of fibrosis. In fact, it is one of the most widely used indicators of fibrosis treatment.

Paracrine effects

Dysregulation of cytokine secretion and signaling is present during the fibrotic process [14]. It is well known that adipose-derived mesenchymal stem cells can secrete a large number of soluble factors[15]. The anti-fibrotic function of human adipose tissue is related to the release of fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF), *etc*. Those growth factors and cytokines are pivotal ways for ASCs to exert their effects, such as pro-angiogenesis and immunomodulation. A study in kidney injury models revealed that an important initiating factor of fibrosis is the loss of capillary bed, which leads to hypoxia, oxidative stress, inflammation and finally, fibrosis. This could be demonstrated by the overlap between low capillary density areas and fibrotic areas. Therefore, since ASCs could secrete multiple pro-angiogenic factors including VEGF, they could alleviate fibrosis by increasing the density of capillaries, therefore relieving hypoxia and ensuing responses [6]. HGF has been demonstrated to downregulate TGF-β1 and modulate the recruitment of immune cells, which plays an essential role in the ASCs anti-fibrotic process[11,16].

Antioxidation

As a result of tissue damage, fibrosis is associated with hypoxic conditions in the affected area, leading to the production of reactive oxygen species (ROS). Fibrotic tissue is characterized by low capillary density and low oxygen concentration[17]. As a result of chronic hypoxia, ROS have a significant effect on the activation of the TGF-β 1/Smad pathway and the accumulation of collagen.

In a study of rat myocardial infarction models, ASCs transplantation significantly decreased ROS level and suppressed the activity of nucleotide-binding oligomerization domain like receptor (NLR) pyrin domain containing 3 (NLRP3) inflammasome, and cardiac fibrosis was markedly ameliorated[18]. At present, many studies have shown that ASCs have powerful antioxidant properties[6,19]. The antioxidative activity of paracrine factors, as well as the improvement in microcirculation due to increased capillary density, leads to a significant reduction in oxidative stress in the

surrounding environment[20].

Immunomodulation

Fibrosis involves the activation of the innate and adaptive immune system[1]. ASCs possess immunomodulatory abilities. Studies have shown that ASCs can reduce inflammation. ASCs play an important role in regulating the function of macrophages, including the conversion from pro-inflammatory M1 subtype to the anti-inflammatory M2 subtype and the recruitment of anti-inflammatory macrophages[21-23]. ASCs also exert powerful immunosuppressive properties by inhibiting T cell response[13]. Mast cells are believed to have a direct effect on stimulating the proliferation of fibroblasts, and the positive results of ASCs scar treatment were associated with a decrease in the number of mast cells in the histological samples of the treatment group[24].

Apart from regulating immune cells, ASCs can also reduce the level of a variety of inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), and IL-6[13]. The changes in fibrosis and possible mechanisms of action of ASCs are summarized in Figure 1.

ALTERED CHARACTERISTICS AND EFFECTS OF ASCS UNDER DISEASE ENVIRONMENTS

Scleroderma

ASCs in a distinct disease microenvironment display different characteristics. Taki *et al* [25] treated ASCs with systemic sclerosis (SSc) lesion tissue fluid, to comprehensively determine the influences of the disease microenvironment on ASCs. Their findings support the idea that ASCs are activated by exposure to the disease microenvironment and can differentiate into fibrotic cells. In addition, subcutaneous ASCs are another potential source of pathogenic myofibroblasts, and multiple factors in the disease microenvironment contribute to the fibrotic transformation (Figure 2).

Metabolic syndrome

Adipose tissue in obesity changes into a pro-inflammatory phenotype, fueled by altered ASCs. They exhibit decreased pluripotency, increased secretion of multiple inflammatory factors, including TNF-α, IL-8, IL-6, *etc*, while the secretion levels of VEGF, FGF, and HGF were reduced. Obese ASCs also synthesize more ROS and recruit more immune cells, continuously promoting the development of inflammation [26]. Over-nutrition also leads to the shortening of telomeres in ASCs, driving them toward cellular senescence, and consequently, fibrosis and functional abnormalities of adipose tissue[27].

The contents of extracellular vesicles (EVs) undergo changes in addition to ASCs themselves. Farahani *et al*[28] conducted microRNA sequencing on the EVs derived from ASCs of lean and metabolic syndrome (MetS) model pigs, and revealed the changes of 19 microRNAs which were related to mitochondria. Further tests on stenotic kidney injury models uncovered that only MetS EVs failed to improve fibrosis or other indicators. Another study also conducted microRNA sequencing on MetS EVs and compared the differentially expressed microRNAs between lean and MetS individuals, both in pig models and human patients. As a result, there were 57 overlaps in differentially expressed microRNAs between pig models and patients, likely related to MetS-induced changes. *In vitro* experiments of co-culturing ASCs with renal tubular cells demonstrated that MetS ASCs could induce senescence in tubular cells. MetS ASCs injection was not able to improve renal fibrosis as effectively as lean ASCs[29].

WAYS TO ENHANCE ASCS THERAPEUTIC PERFORMANCE

Pre-conditioning

Multiple studies have shown that pre-conditioning with chemicals or protein factors could enhance the therapeutic performance of ASCs. Antioxidants are common sought choices. Liao *et al*[30] pre-treated ASCs with reduced glutathione (GSH) or melatonin, both of which are endogenous antioxidants. Pre-treatment enhanced the anti-fibrosis and anti-inflammatory effects of transplanted ASCs due to promoted migration and survival. The enhanced survival of ASCs could result from increased expression of Bcl-

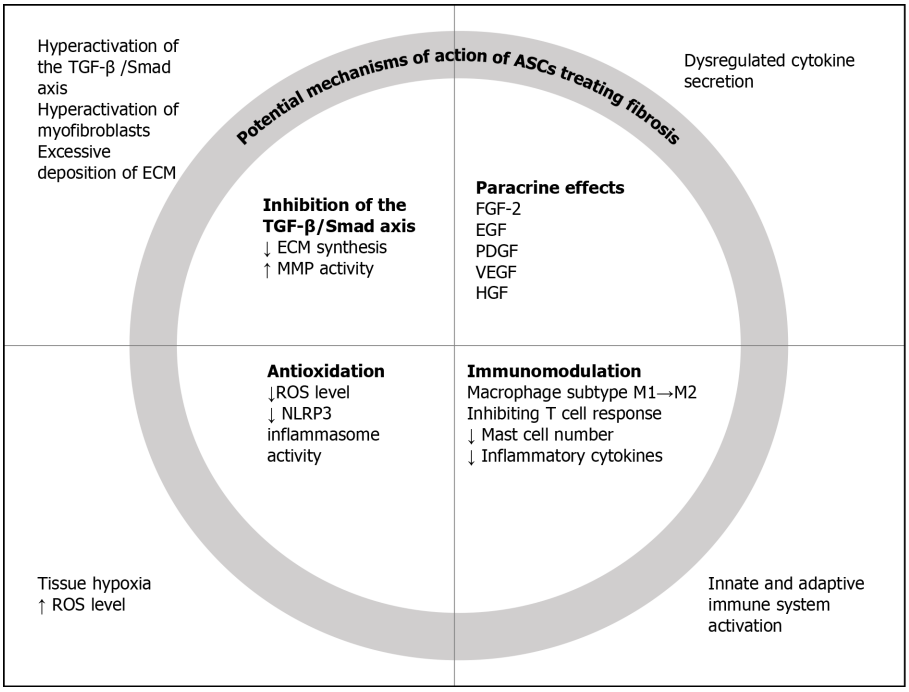

Figure 1 Changes in fibrosis and the potential mechanisms of action of ASCs. TGF-β: Transforming growth factor-β; ECM: Extracellular matrix; MMPs: Matrix metalloproteinases; FGF-2: Fibroblast growth factor-2; EGF: Epidermal growth factor; PDGF: Platelet-derived growth factor; VEGF: Vascular endothelial growth factor; HGF: Hepatocyte growth factor; ROS: Reactive oxygen species; NLRP3: Nucleotide-binding oligomerization domain like receptor pyrin domain containing 3.

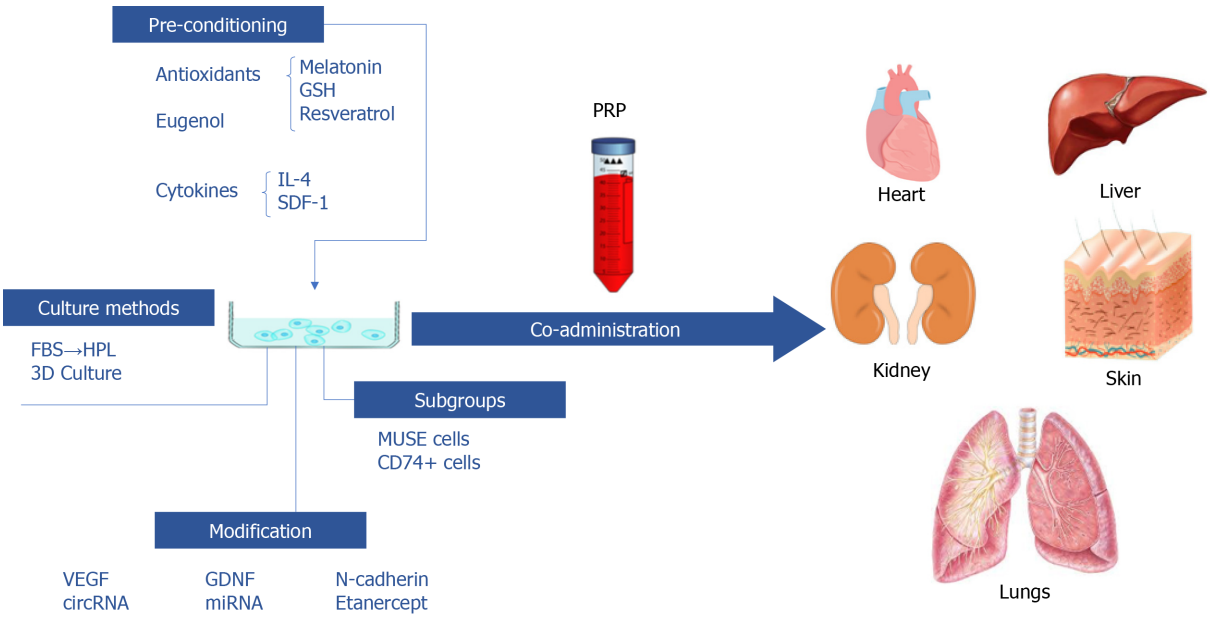

Figure 2 Ways to enhance adipose-derived stem cells therapeutic performance. GSH: Glutathione; IL-4: Interleukin 4; SDF-1: Stromal derived factor-1; FBS: Fetal bovine serum; HPL: Human platelet lysate; 3D culture: Three-dimensional culture; ECM: Extracellular matrix; VEGF: Vascular endothelial growth factor; GDNF: Glial cell line-derived neurotrophic factor; circRNA: Circular RNA; miRNA: micro RNA; MUSE cells: Multilineage differentiating stress enduring; PRP: Platelet-rich plasma.

2 and Cyclin-D1 and reduced expression of Bax, that is, antioxidant pre-treatment promoted cell proliferation and reduced apoptosis. *Ex vivo* imaging showed that GSH and melatonin pre-treatment promoted ASCs migration into the liver, and allowed them to maintain at a relatively high level. Further investigation revealed that the amelioration of ROS-induced oxidative stress might be the underlying mechanism of action of the antioxidants, and that GSH might be a better choice of pre-conditioning compared to melatonin. Resveratrol is a natural antioxidant extracted from plants

which has been proved to benefit health and enhance stem cells therapeutic performance. Chen *et al*[5] pretreated ASCs with resveratrol (RSVL-ASCs). Compared to untreated ASCs, RSVL-ASCs exhibited a stronger anti-fibrotic effect in diabetes mellitus cardiomyopathy.

Fathy *et al*[31] discovered that eugenol pre-treatment enhanced ASCs' self-renewal, proliferation and migration abilities *in vitro*, as well as their therapeutic effects in rat liver fibrosis models. Compared to untreated ASCs, eugenol treated ASCs (E-ASCs) exhibited better homing ability, further decreased the expression level of inflammatory factors and increased MMPs expression. Histopathological examination revealed similar results.

Zimowska *et al*[32] studied whether ASCs treated with IL-4 and stromal derived factor-1 (SDF-1), both of which enhance skeletal muscle regeneration, had a better effect in promoting skeletal muscle regeneration. *In vitro* treatment of IL-4 and SDF-1 significantly enhanced ASCs proliferation and migration.

Pre-conditioning with chemicals and protein factors proved to be effective in improving the functions of ASCs. However, since there could be residual substances in treated cells, it is pivotal to test and determine their safety before application.

Transfection and modification

Modifying ASCs with various factors could boost their therapeutic effects or expand their mechanisms of action. VEGF is an essential factor in promoting angiogenesis, and is naturally secreted by ASCs. Boosting the concentration of VEGF might result in a boosted therapeutic effect. Yu *et al*[7] studied the pro-angiogenic, anti-fibrotic and ability to improve fat graft survival rate of VEGF mRNA modified ASCs *in vitro* and *in vivo*. Compared to unmanipulated controls, VEGF mRNA modification greatly amplified the therapeutic effects, likely due to the increased secretion of VEGF. Glial cell line-derived neurotrophic factor (GDNF) is a neuroprotective factor that has also been proved to play a critical role in kidney diseases. Li *et al*[6] transfected GDNF gene into ASCs in order to explore whether the modified cells could perform better in treating renal interstitial fibrosis. It was discovered that the modified ASCs secreted more growth factors, and possessed enhanced abilities in many aspects.

Apart from classical growth factors, N-cadherin is a transmembrane protein that can enhance cell-cell adhesion which has only recently attracted attention in the field of stem cell biology. In a recent study[33], adult mice-ASCs were transfected with adenovirus harboring N-cadherin. N-cadherin overexpression promoted the migration and angiogenic properties, and significantly increased the formation of the N-cadherin/β-catenin complex and the level of active β-catenin in the nucleus, which leads to increased expression levels of MMP-10, MMP-13, and HGF, thus exerting their anti-fibrosis effect.

Another method of exploration is to first compare the differences between disease and normal tissues, identify the genes most likely in play, and adjust ASCs with pertinency. Zhu *et al*[34] compared the circular RNA (circRNA) expression profiles of normal and fibrotic liver samples from CCl_4 induced liver fibrosis mouse models, and discovered that mmu_circ_0000623 was downregulated in fibrotic liver samples. Further exploration revealed that mmu_circ_0000623 interacted with miR-125/ATG4D and modulated autophagy. ASCs were modified with mmu_circ_0000623 and their exosomes were collected. Exosomes from modified ASCs resulted in the best anti-fibrotic effect *in vitro* and *in vivo*, by regulating autophagy.

Micro RNAs (miRNAs, or miRs) are small non-coding RNA molecules (about 22 nucleotides) that change gene expression at the post-transcriptional level, leading to changes in protein synthesis. MiR-150 is a representative anti-fibrotic miRNA, which can inhibit the activation of hepatic stellate cells through the inhibition of C-X-C motif chemokine ligand 1 (CXCL1), and is a natural component of ASCs EVs[35]. Paik *et al*[36] transfected ASCs with miR-150 to explore whether additional miR-150 secretion could boost the anti-fibrotic ability. As expected, transfected ASCs better alleviated liver fibrosis both *in vitro* and *in vivo*, due to elevated secretion of anti-fibrotic miRs[36].

TNF-α-driven inflammation plays a key role in the occurrence of liver fibrosis. Han *et al*[37] designed genetically engineered ASCs that can produce etanercept (an effective TNF-α inhibitor) to play an anti-fibrosis role. They transfected ADSCs with a microcirculatory plasmid containing an insert encoding the etanercept gene to generate synthetic etanercept ADSCs. The results showed that blocking TNF-α-driven inflammation at the appropriate stage of liver fibrosis with the advantage of ADSCs may be an effective strategy to prevent fibrosis.

In summary, genetically manipulated ASCs could be useful in adjusting to different disease conditions and boosting therapeutic effects. However, great care must be taken when considering administrating them into humans. It is crucial to first make sure that the manipulated cells are not tumorigenic or in other ways harmful.

Culture methods

2-dimensional culture remains the primary approach to expand ASCs *in vitro*. However, various materials used in this process increased the risk of contamination and immune rejection. For example, xenogeneic fetal bovine serum (FBS) might induce immune reactions or transmit bovine diseases. Human platelet lysate (HPL) has been proved to be a promising substitute for FBS. Replacing FBS with HPL in ASCs expansion medium resulted in better proliferative ability without changing cell surface markers. Although TGF-β1-stimulated fibroblasts exhibited improved migration upon HPL-cultured ASCs conditioned medium treatment, there were marked reductions in TGF-β1 and alpha-smooth muscle actin (α-SMA) expression, indicating that the anti-fibrotic ability was likely enhanced by HPL culturing. Further investigation by adding the HGF neutralizing antibody revealed that the anti-fibrotic effect was at least partially related to the increased secretion of HGF[11]. This well-rounded study explored the feasibility of substituting FBS with HPL, and paved the way for safer clinical application of ASCs.

At present, 3-dimensional (3D) cell culture is a frequently-used method to enhance stem cell functions. Transglutaminase cross-linked gelatin (Col-Tgel) is a stiffness-tunable cell culture medium which exhibits excellent performance in cellular adhesion and proliferation and can release entrapped cells. ASCs and Col-Tgel were co-cultured in an *in vitro* 3D system. Compared with ASCs alone, Col-Tgel embedded ASCs significantly enhanced the long-term retention rate and cardioprotective effect of ASCs in acute myocardial infarction models[2].

Aside from Col-Tgel, various 3D culture methods await exploration. Different target diseases and application methods shall require matching characteristics of the culture medium. ECM mechanical characteristics could affect ASCs phenotype and secretion of cytokines. It has long been known that matrix stiffness is an important influencing factor that affects stem cells lineage specification[38]. Dunham *et al*[39] discovered that culturing ASCs in a stiff matrix resulted in a more pro-fibrotic phenotype, including increased actin and α-SMA expression and ECM secretion. An adipokine array revealed altered expression levels of multiple adipokines, of which endocan, insulin-like growth factor-binding protein-6 (IGFBP-6), and monocyte chemoattractant protein-1 (MCP-1) were increased in the stiff matrix and might play a part in environment stiffness induced changes of ASCs. *In vivo* experiments in post-traumatic elbow contracture rat models revealed similar results: ASCs cultured in soft matrix had the best therapeutic effects in increasing elbow range of motion and fibrosis, while ASCs cultured on tissue culture plastic surface made little difference. It was interesting that ASCs transferred from soft matrix into stiff matrix exhibited mechanical memory and maintained the soft matrix-induced phenotypes for a week, which may provide new methods for enhancing the therapeutic effects while avoiding adverse effects of ASCs.

Apart from stiffness, matrix microarchitecture is another factor that influences ASCs characteristics. Seo *et al*[40] investigated the effects of collagen microarchitecture on ASCs differentiation towards myofibroblasts. ASCs were cultured in collagen fibers that were different in diameter and pore size. Increased contractility, myofibroblast differentiation and pro-angiogenic phenotype were detected in ASCs cultured in thicker fibers and larger pores. The contractile ASCs in turn re-arranged local collagen fibers, therefore increasing local stiffness, and reciprocally, environment stiffness would eventually act on ASCs, forming a circuit of action. Notably, these changes were independent of collagen concentration and bulk stiffness.

ASCs subgroups

There are subgroups within ASCs that possess varied abilities. Multilineage differentiating stress enduring (MUSE) cells are thought to be early-stage MSCs. They can be sorted by severe cellular stress conditions or flow cytometry, using the markers stage-specific embryonic antigen 3 (SSEA-3) (a marker of human ES cells) and endoglin (CD105) (a marker of MSCs). Enhanced pluripotency, paracrine effects, high homing ability and low tumorigenicity have been observed in MUSE cells compared to unsorted ASCs[41].

Borrelli *et al*[42] identified a subpopulation of ASCs that were positive for CD74. They proved to have enhanced anti-fibrotic abilities both *in vitro* and *in vivo*. CD74+ ASCs conditioned medium possessed stronger anti-fibrotic ability, possibly through

elevated secretion of HGF, FGF2, TGF-β3 and decreased level of TGF-β1. In radiation-induced fibrosis mouse models, CD74+ ASCs assisted fat graft most significantly, reduced dermal thickness and fibrosis, and resulted in the highest fat survival rate.

As ASCs are inherently heterogenous, subgroups are worth studying in the hope of discovering suitable subgroups for different diseases and the cells that possess maximum healing powers and minimum possibility of inducing adverse effects.

Co-administration

In addition to focusing on ASCs themselves, another way of improving therapeutic effects is the combined usage of ASCs and other substances. Evin *et al*[3] co-administered ASCs with platelet-rich plasma (PRP) in treating radiation-induced fibrosis. The combination of ASCs and PRP rendered the best results, providing evidence that PRP could augment the therapeutic effects of ASCs.

APPLICATION IN FIBROSIS-RELATED DISEASES

Muscles

During muscle repair, fibrosis is a common adverse factor as it stiffens and weakens the healed muscle, depriving it of normal function. *In vivo* transplantation of ASCs into skeletal muscle injury models resulted in better muscle morphology under the microscope, with more regenerated myofibers and less collagen fiber deposition, through regulating immune responses[32]. It is worth noting that ASCs exhibited better regenerative effects than bone marrow-derived mesenchymal stem cells[43], providing evidence for choosing the most suitable cell type for muscle repair.

Many heart diseases are attributed to cardiac fibrosis and remodeling. Clinically, thrombolytic therapy and primary percutaneous coronary intervention are the most effective treatments for acute myocardial infarction (MI), but due to the limited heart regeneration capacity of adult mammals, irreversible heart failure cannot be prevented. It is expected that stem-cell based regenerative therapy could benefit cardiac fibrosis after infarction. Yan *et al*[33] aimed to explore whether and how N-cadherin (NCAD) regulates mesenchymal stem cell retention and cardio-protection against ischemic heart failure (IHF). It was found that ASCs transfected with NCAD significantly increased mouse left ventricular ejection fractions and reduced fibrosis. NCAD overexpression can promote ASCs-cardiomyocyte adhesion and migration, and enhance their angiogenesis and cardiomyocyte proliferation abilities. They confirmed for the first time that NCAD overexpression can mediate the expression and production of MMP-10/MMP-13/HGF through β-catenin to enhance the protective effect of ASCs on IHF.

Chen *et al*[2] delivered the aforementioned Col-Tgel enwrapped ASCs into the heart muscle of acute MI models. The results showed that Col-Tgel provided a suitable microenvironment for the survival, proliferation, and migration of ASCs into the ischemic myocardial tissue, which is essential to exert their regenerative and anti-fibrotic effect. This method can be used clinically to improve the effective rate and reproducibility of cell therapies and heart regeneration research.

EVs deliver genes and proteins to recipient cells and mediate the paracrine activity of their parent cells, which also plays a role in the cardioprotective effect of ASCs. In a pig model with both MetS and renal artery stenosis (RAS), intrarenal injection of EVs derived from ASCs reduced the release of pro-inflammatory cytokines. In MetS+RAS patients, intrarenal injection of EVs reduced myocardial damage, which could be related to the improvement in renal function and systemic inflammation. Local concentrations of inflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1), TNF-α, and IL-6 were reduced in the stenotic kidney. EVs derived from ASCs improved myocardial fibrosis and remodeling, reduced myocardial hypoxia, improved capillary density and microvascular function, and reduced myocardial cell senescence, thus improving the diastolic function[44].

The efficacy of ASCs has been known to clinicians for some time. Trials have been conducted to explore whether ASCs can safely improve cardiac fibrosis after MI. The results were satisfactory, and were thoroughly elaborated by Li *et al*[45] and Vazir *et al*[46].

Although numerous studies support the effectiveness of ASCs in treating cardiac fibrosis, negative results exist where ASCs are not as effective as induced pluripotent stem cell-derived cardiomyocytes[47]. Therefore, it is necessary to make comparisons to understand both the advantages and the disadvantages of ASCs.

Liver fibrosis

Liver fibrosis is an excessive wound healing process that occurs in response to liver injury depending on the underlying cause. Currently, there are no effective treatments for liver fibrosis other than liver transplantation. Due to their advantages over stem cells from other sources, ASCs have received extensive attention as regenerative drugs for the treatment of liver fibrosis[35,48].

Intravenously injected ASCs can migrate into and survive in the fibrotic liver in animal models. Fibrosis was significantly reduced 3 wk after ASCs injection. Immunohistochemistry assay indicated that ASCs had the potential to differentiate into hepatic cells *in vivo*[48].

However, the inherent limitations of stem cell therapy, such as cell rejection and possibility of tumor formation, hinder the clinical application of ASCs-based therapy. To overcome these problems, extracellular nanovesicles (ENVs) responsible for the treatment of ASCs (A-ENVs) have shown considerable promise as a cell-free treatment of liver diseases. Han *et al*[49] studied the *in vivo* and *in vitro* anti-fibrotic effects of A-ENVs in a thioacetamide-induced liver fibrosis model. A-ENVs significantly downregulated the expression of fibrogenesis markers, such as MMP-2, collagen-1, and α-SMA. Systemic injection of ENVs can accumulate in fibrotic liver tissue and restore liver function. These results demonstrate the great potential of A-ENVs as a therapeutic method based on ENVs in the treatment of liver fibrosis and possibly other difficult chronic liver diseases.

ASCs could also exert their anti-fibrotic function through an EV component, miR-150-5p and its downstream target, CXCL1, which is related to hepatic stellate cells activation and ensuing fibrosis. Du *et al*[35] demonstrated that the EVs from ASCs contain miR-150-5p, and EVs treatment downregulated CXCL1 expression in hepatic stellate cells, preventing their activation. *In vivo* experiments showed reduced fibrosis and inflammation in the EVs treated group that correlated with the inhibition of CXCL1.

With the developing studies on ASCs derivatives, a question naturally arises: are the derivatives as effective as ASCs themselves? Watanabe *et al*[21] established non-alcoholic steatohepatitis (NASH) models in mice, tested and compared the therapeutic effects of MSCs and their small EVs. Liver fibrosis was significantly reduced after MSCs or sEVs treatment. In terms of relieving fibrosis, the effect of 5.0 μg of sEVs was equal to that of 1×10^6 MSCs, indicating that although ASCs derivatives possess several advantages compared to the direct application of ASCs themselves, a certain amount or concentration would have to be reached for them to have a satisfactory effect.

In order to enhance treatment effectiveness, various modifications were made, including the aforementioned antioxidant and eugenol pre-treatment, modification with various factors including mmu_circ_0000623 and etanercept, all exhibited enhanced abilities in treating liver fibrosis[30,31,34,37].

Attempts have been made in terms of applying ASCs in treating liver fibrosis patients. Huang *et al*[50] treated liver cirrhosis with GXHPC1, a cell product that contains human ASCs in a phase I clinical trial. Previous trials in animal models proved GXHPC1 to be effective and safe. In their clinical trial, intrahepatic injection of GXHPC1 did not cause any safety problems. The liver function and quality of life of liver cirrhosis patients was improved significantly.

Kidney diseases

Fibrosis appears in various kidney diseases, and is an indicator of the degree of renal structural damage. Intravenously injected ASCs can migrate into the injured kidney in renal interstitial fibrosis mouse models, promote angiogenesis through the PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)/AKT (v-akt murine thymoma viral oncogene homologue) pathway, alleviate oxidative stress and thus significantly reduce renal fibrosis[6]. RAS can lead to kidney ischemia and injury. Kim *et al*[51] demonstrated that this injury was related to cellular senescence, and that ASCs effectively reversed RAS-induced kidney injury and fibrosis, partly through the alleviation of cellular senescence.

A comparison between ASCs and their derivatives was also conducted in kidney diseases. ASCs and their EVs can both attenuate kidney injury, while focusing on different aspects. Both of them improved kidney fibrosis, hypoxia and cellular apoptosis levels, but the cells were better at increasing capillary density and reducing inflammation, yet their EVs were more efficient in preserving the integrity of kidney cells[52]. The differences represent various components and mechanisms awaiting exploration, which may guide future therapeutic choices.

Apart from kidney fibrosis itself, prolonged peritoneal dialysis (PD) can result in peritoneal fibrosis (PF), a long-term complication jeopardizing peritoneal membrane (PM) function. An intravenous injection of 1×10^6 ASCs proved to not only block the development of PF, but also alleviate the fibrosis and inflammation of already formed PF in rat models of chronic kidney disease (CKD) combined with PF. ASCs treatment significantly downregulated the expression levels of IL-1β, TNF-α, and IL-6, and avoided macrophage and T-cell infiltration into the PM in chlorhexidine gluconate induced PF in CKD rats[13].

Nevertheless, we should recognize the fact that ASCs are not a panacea. Chen *et al*[19] discussed whether adjunct ASCs could facilitate shockwave therapy in treating atherosclerotic renal artery stenosis (ARAS). Masson trichrome-staining exhibited a similar degree of fibrosis in shockwave therapy alone or shockwave + ASCs; thus, adjunct ASCs did not further improve fibrosis in ARAS. However, adjunct ASCs did have better performance in retaining more capillaries and reducing oxidative stress, which is clearly beneficial to the treatment of kidney diseases.

Skin

One of the major differences between normal and fibrotic skin is that the collagen fibers are randomly oriented in normal skin, while arranged in large bundles in fibrotic skin, causing it to thicken and stiffen[53]. Various conditions involve fibrosis of the skin, such as hypertrophic scars, radiation-induced fibrosis, and scleroderma, *etc*.

Hypertrophic scarring is caused by hyperactivation of fibroblasts and excessive accumulation of extracellular matrix during wound healing. Studies have shown that ASCs are capable of preventing hypertrophic scar occurrence and progression[10,54]. It has also been observed that ASCs themselves are more effective than their conditioned medium, possibly due to insufficient concentration of paracrine factors and lack of cell-cell contact induced regulation which would allow ASCs to react according to their surrounding environment[55].

Since the concentration of paracrine factors in conditioned medium is rather low, lyophilizing is an effective way to condense. Freeze-dried ASCs conditioned medium powder could reduce hypertrophic scar fibroblasts activity *in vitro* in a dose-dependent manner. In rabbit ear hypertrophic scar models, topical administration of lyophilized ASCs conditioned medium significantly reduced hypertrophic scarring. The effect is most significant when combining the conditioned medium powder with polysaccharide hydrogel, which provided a medium for the sustained release and continuous action of paracrine factors[10].

Other skin fibrotic diseases, for example, radiation-induced fibrosis, is not uncommon among patients receiving radiotherapy. Subcutaneous injection of ASCs alleviated fibrosis, along with other skin complications caused by radiation, and the therapeutic effect was amplified by PRP co-administration[3].

SSc is an autoimmune disease that often involves the skin and lungs. Okamura *et al*[56] found that ASCs may prove to be a potential therapeutic drug for SSc patients. Administration of ASCs alleviated skin and lung fibrosis of bleomycin-induced scleroderma and sclerodermatous chronic graft-versus-host disease (Scl-cGVHD) model mice. Experimental results showed that ASCs inhibited the infiltration of CD4+ T cells, CD8+ T cells, and macrophages in the dermis of bleomycin model mice and reduced the mRNA levels of collagen and fibrotic cytokines, such as IL-6 and IL-13.

Fat graft is a frequently used method for treating fibrotic skin diseases such as scleroderma. However, the low retention rate had always been a troubling issue. Zhu *et al*[23] applied ASC-EVs to fat grafts in mice, and measured fat graft survival rate at up to 12 wk post-surgery. Not only did ASC-EVs improve fat retention, they also altered the ratio of M1/M2 macrophages toward an anti-inflammatory state, promoted the browning of white adipose tissue, and reduced fibrosis in fat grafts.

Ogino *et al*[53] studied the therapeutic effects of ASCs transplantation in secondary lymphedema, where chronic accumulation of tissue fluid often leads to skin fibrosis. Picrosirius red staining revealed that ASCs restored type I collagen orientation and increased type III collagen content, thus relieving dermal fibrosis in lymphedema mice. Promoted lymphangiogenesis was observed, which is pivotal to the alleviation of tissue edema, thereby blocking the development of dermal fibrosis. Since there is a lack of effective treatment methods for lymphatic diseases, ASCs serve as a promising treatment modality due to their multifaceted functions.

In terms of clinical trials, ASCs were used to treat post-acne scars and SSc (Table 1). The hand disability cause by SSc skin lesions is a tricky problem that has significant impact on patients' quality of life and is difficult to treat. As multiple preclinical studies have demonstrated the anti-fibrosis effect of ASCs, attempts were made to apply ASCs in the treatment of SSc hand disability. Subcutaneous injection of stromal

Table 1 Clinical trials of adipose-derived stem cells treating skin fibrotic diseases

Trial number	Ref.	Disease	Study type	Study population	Cell/derivative type	Amount	Method of administration	Outcome measurement	Follow-up period	Results	Complications
NCT03060551	Park *et al*[58], 2020	SSc hand disability	Open-label, single center clinical trial	18	SVF	3.61×10^6 each finger on average	Subcutaneous injection	Skin fibrosis, hand edema, hand disability, severity of Raynaud's phenomenon and hand pain, quality of life, active ulcers, nailfold capillary microscopy	6 mo	Improved skin fibrosis, edema, quality of life, as well as other aspects	No serious adverse events occurred. Five minor adverse events were reported, including paresthesia in liposuction area, dizziness after lidocaine injection, and transient pallor that soon resolved without sequelae
NCT01813279	Granel *et al* [57], 2014; Guillaume-Jugnot *et al* [59], 2015	SSc hand disability	Open-label, single arm phase I trial	12	SVF	$3.76 \pm 1.85 \times 10^6$ each finger	Subcutaneous injection	Hand disability, fibrosis, vascular manifestations, pain and quality of life	12 mo	Significant improvements inskin sclerosis, hand function, finger edema and quality of life that lasted for at least a year	No serious adverse events occurred. Four minor adverse events were reported that spontaneously resolved
Not applicable	Abou Eitta *et al*[65], 2019	Post-acne scars	A single-center, split-face, prospective clinical trial	10	SVF	6×10^6 on average	Subcutaneous injection	The global scoring system, TEWL and skin hydration	3 mo	A significant improvement in the degree of scar severity, scar area percent, skin hydration, and TEWL	Not mentioned
Not applicable	Zhou *et al*[66], 2016	Facial atrophic acne scars and skin rejuvenation	A single-center, split-face, prospective clinical trial	22	ASC-CM	3 mL	Topical application on laser treated sites, 3 sessions at one-month intervals	The subjective satisfaction scale, improvement score, biophysical measurements, and skin biopsies	3 mo	Topical application of ASC-CM can increase the efficacy of FxCR treatment of atrophic acne scars and skin rejuvenation, while simultaneously reduces adverse reactions post laser therapy	No complications reported

SSc: Systemic sclerosis; mRSS: Modified Rodnan skin score; ASC-CM: Conditioned medium of adipose-derived stem cells; FxCR: Fractional carbon dioxide laser resurfacing; TEWL: Trans-epidermal water loss; SVF: Stromal vascular fraction.

vascular fraction (SVF) greatly improved fibrosis, hand function, quality of life and other aspects[57-59]. ASCs might be the long-expected solution for this debilitating condition.

Lung fibrosis

Lung fibrosis is a debilitating condition that can occur in many diseases. Current therapies are insufficient, thus posing the demand for better solutions, such as stem cell therapy[60].

Baer *et al*[61] transplanted ASCs isolated from luciferase transgenic mice (mASCs) into Atm-deficient mice which mimic the lung injury in human Ataxia-telangiectasia syndrome. Using an *in vivo* bioluminescence imaging (BLI) system, they found that the intravenously injected ASCs migrated into the injured lungs of mouse models, and were present for up to 9 to 14 d. Since the *in vivo* disposition of transplanted ASCs is not yet completely clear, BLI might serve as a powerful tool for tracking the whereabouts of cells, providing crucial information regarding the safety of cell therapy.

A comparison between mesenchymal stem cells (MSCs) from different sources was conducted in mouse lung fibrosis models. Among stem cells from adipose tissue (ASCs), Wharton's jelly (WJ-MSCs), chorionic membrane (CSCs) and chorionic villi (CVCs), ASCs proved to be the most effective and well-rounded in different therapeutic aspects[60]. Another comparison was made in pulmonary hypertension (PAH) models. Mesenchymal stem cells from adipose tissue (ASCs), bone marrow (BMSCs) and umbilical cord blood (UCB-MSCs) were compared. As a result, UCB-MSCs proved to be the most effective in treating PAH, exhibiting the greatest improvement in cardiac function as well as reductions in fibrosis, inflammation, and classic PAH pathways[62]. It is interesting that MSCs from different sources vary so much in their effects. Further research is needed to investigate the mechanisms involved in order to understand and find the best match between diseases and therapeutic cell types.

The functions and mechanisms of ASCs are not singular, but rather intricate. Lim *et al*[12] found that intravenously injected ASCs migrated into the lungs of murine Scl-GVHD models, but not into the skin. It seems that ASCs exhibited a pro-inflammatory effect in the lungs: CD11b monocyte/macrophages and CD4 T cells were recruited, expression levels of CC chemokine 1 (CCL1) and multiple chemokines were upregulated, and a deterioration in pathological score was observed. Moreover, blocking CCL1 exerted protective effects, relieving inflammation and fibrosis in the lungs. However, despite the possibly detrimental role ASCs play in the lungs, they exhibited anti-inflammatory and anti-fibrotic effects in the skin, which were preserved, or even enhanced, after CCL1-blocking antibody treatment. The fact that ASCs alleviated skin fibrosis while exacerbating lung injuries in Scl-GVHD posed a potential threat to the safety of ASCs therapy. However, CCL1-blocking antibody treatment could avoid detrimental effects while preserving or even enhancing the protective effects which offered a solution. The combination of stem cells and CCL1-blocking antibody provides a new option in exploiting the therapeutic effects of ASCs while avoiding the possible adverse effects.

Not all studies yielded positive healing effects. A study in acute respiratory distress syndrome (ARDS) mouse models revealed that ASCs treatment inhibited the recruitment of neutrophils, reduced short-term lung injury, and alleviated long-term fibrosis. However, the level of inflammatory cytokines did not decrease significantly, the therapeutic effect was minimal and not clinically significant[63].

Despite the possibly unstable effects shown in preclinical studies, a clinical trial of ASCs treating lung fibrosis yielded positive results. Tzouvelekis *et al*[64] treated idiopathic pulmonary fibrosis with ASCs-SVF in a phase Ib clinical trial, in order to prove the safety of ASCs treatment. There were no serious adverse events, and functional parameters and quality of life indicators did not deteriorate.

CONCLUSION

ASCs are promising candidates for the treatment of various fibrotic diseases. Multiple methods could be exploited in order to boost the therapeutic effects of ASCs. However, the functions of ASCs are somewhat indeterminate and complicated. The effectiveness and safety issues in ASCs therapy, and the most matching diseases suitable for ASCs therapy remain to be explored.

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ORIGINAL ARTICLE

Basic Study

Exosomes derived from inflammatory myoblasts promote M1 polarization and break the balance of myoblast proliferation/differentiation

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Abstract

BACKGROUND

Acute muscle injuries are one of the most common injuries in sports. Severely injured muscles are prone to re-injury due to fibrotic scar formation caused by prolonged inflammation. How to regulate inflammation and suppress fibrosis is the focus of promoting muscle healing. Recent studies have found that myoblasts and macrophages play important roles in the inflammatory phase following muscle injury; however, the crosstalk between these two types of cells in the inflammatory environment, particularly the exosome-related mechanisms, had not been well studied.

AIM

To evaluate the effects of exosomes from inflammatory C2C12 myoblasts (IF-C2C12-Exos) on macrophage polarization and myoblast proliferation/differentiation.

METHODS

A model of inflammation was established *in vitro* by lipopolysaccharide stimulation of myoblasts. C2C12-Exos were isolated and purified from the supernatant of myoblasts by gradient centrifugation. Multiple methods were used to identify the exosomes. Gradient concentrations of IF-C2C12-Exos were added to normal macrophages and myoblasts. PKH67 fluorescence tracing was used to identify the interaction between exosomes and cells. Microscopic morphology, Giemsa stain, and immunofluorescence were carried out for histological analysis. Additionally, ELISA assays, flow cytometry, and western blot were conducted to analyze molecular changes. Moreover, myogenic proliferation was assessed by the BrdU

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

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test, scratch assay, and CCK-8 assay.

RESULTS
We found that the PKH-67-marked C2C12-Exos can be endocytosed by both macrophages and myoblasts. IF-C2C12-Exos induced M1 macrophage polarization and suppressed the M2 phenotype *in vitro*. In addition, these exosomes also stimulated the inflammatory reactions of macrophages. Further-more, we demonstrated that IF-C2C12-Exos disrupted the balance of myoblast proliferation/differentiation, leading to enhanced proliferation and suppressed fibrogenic/myogenic differentiation.

CONCLUSION
IF-C2C12-Exos can induce M1 polarization, resulting in a sustained and aggravated inflammatory environment that impairs myoblast differentiation, and leads to enhanced myogenic proliferation. These results demonstrate why prolonged inflammation occurs after acute muscle injury and provide a new target for the regulation of muscle regeneration.

Key Words: C2C12 myoblast; Exosomes; Macrophage polarization; Inflammation; Differentiation; Proliferation

Core Tip: For successful muscle regeneration, macrophage polarization and myogenesis should be supported by an appropriate combination of cells and their signals. As the communication between myoblasts and macrophages within the inflammatory environment is unknown, we aimed to evaluate the effects of IF-C2C12-Exos on macrophage polarization and myoblast proliferation/differentiation. We found that IF-C2C12-Exos could induce M1 polarization, resulting in a sustained and exacerbated inflammatory environment, impaired myoblast fibrogenic/myogenic differentiation, and led to abnormal myogenic proliferation. These results indicate a potential mechanism for the development of long-term inflammation following acute muscle injury, but further preclinical evaluations targeting IF-C2C12-Exos in animal models are necessary.

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INTRODUCTION

More than half of sports injuries in athletes have been reported to be related to muscle damage[1]. In addition to promoting muscle regeneration, preventing scar formation is a key factor in the healing of injured muscle[2-4], as localized fibrotic tissue can lead to susceptibility to re-injury of injured muscles[5,6].

Skeletal muscle fibrosis is related to excessive local inflammation and myoblast fibrogenic differentiation in the early stage after injury[7-11]. During the general inflammatory process following injury, M1 macrophages (classically activated) are recruited and lead to further muscle damage[12], which are then substituted by M2 macrophages (alternatively activated) to promote muscle regeneration and differentiation[13]. Sometimes, M2 polarization is suppressed by the excessive inflammatory conditions that follow acute muscle injury[14,15]. Given the timing of the appearance of M1 coupled with that of myoblasts within 1-3 d after muscle injury[12,16], we suppose that myoblasts may be able to influence the shift from M1 macrophages to M2 subtype. Periodontal ligament stem cells and adipocytes within the inflammatory environment have been reported to inhibit the M2 polarization of macrophages through exosomes[17,18]. Many species of living cells such as myoblasts secrete

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exosomes that can be transported and regulate the essential cell activities such as proliferation and differentiation of neighboring cells[19-22]. Therefore, we speculated that in the prolonged inflammatory micro-environment of injured muscle, myoblasts might promote M1 macrophage polarization and suppress M2 phenotype through exosomes.

Additionally, Guescini *et al*[23] reported that exosomes derived from myotubes can influence the balance of proliferation and differentiation of normal myoblasts after H_2O_2 administration, suggesting that the C2C12-Exos could also regulate muscle healing through self-control[23]. In our previous studies, we found that myoblasts could be converted to myofibroblasts after muscle injury due to its intracellular signals including lncRNA-MFAT1/H19, miR-122-5p/25-3p, and transforming growth factor-β (TGF-β)/SMAD[24-26], which may contribute to muscle fibrosis. Therefore, it is worth investigating whether C2C12-Exos could influence the fibrogenic/myogenic differentiation and proliferation of normal myoblasts.

In this study, we isolated C2C12-Exos from myoblasts within an inflammatory environment, tested their functions, and investigated the potential underlying mechanisms of the effects of C2C12-Exos on the immunomodulation of macrophages and myoblasts proliferation/differentiation *in vitro*.

MATERIALS AND METHODS

Cell culture

C2C12 myoblasts (murine cell line), purchased from ScienCell Research Laboratories, were maintained in high glucose Dulbecco's modified eagle medium (DMEM) (HyClone) with 10% fetal bovine serum (FBS) and 0.5 mL of penicillin/streptomycin solution (#0503; ScienCell Research Laboratories) in a humidified incubator at 37°C and 5% CO_2 atmosphere. Muscle differentiation was induced using 2% horse serum (ThermoFisher) for C2C12. Lipopolysaccharide (LPS), at a concentration of 1000 ng/mL was used to induce an inflammatory environment for C2C12. The medium was then washed three times to remove all the LPS and a fresh exosome-depleted medium was added. C2C12 conditioned media (C2C12-CM) were collected using a 1 mL pipette and added into 50 mL tubes after 24 h incubation. The media were then kept at -80°C before use. Exosomes from C2C12 myoblasts (C2C12-Exos) were extracted by the following steps.

RAW264.7 cells (mouse leukemia cells of monocyte-macrophage), purchased from the American Type Culture Collection, were cultured in DMEM containing 10% FBS and 0.5 mL of penicillin/streptomycin solution in a humidified incubator at 37°C and 5% CO_2 atmosphere.

Isolation and identification of C2C12-Exos

The extraction procedure for C2C12-Exos was based on a previous method[27]. 50 mL conditioned culture medium containing 5 mL exosome-free FBS (Exosome-depleted FBS, Gibco), and 0.5 mL of penicillin/streptomycin solution were used to culture C2C12s for 48 h. After the C2C12s had grown to more than 90% confluence, the cells were treated with LPS for 1 d. Then, fresh culture medium was added, and the cells were kept quiescent for 24 h, and then all the supernatant was collected. After that, all media were subjected to sequential centrifugation (Optima XPN-100 ultracentrifuge; Beckman Coulter SW 41 Ti rotor) at 10000 ×g for 35 min (to remove the cell debris) and then at 100000 ×g for 70 min. After this step, at 100000 ×g for 70 min the precipitate was washed twice with phosphate-buffered saline (PBS). The C2C12-Exos were resuspended in PBS and stored at -80°C prior to processing.

To observe the morphology of exosomes, C2C12-Exos were assessed directly under transmission electron microscopy (Tecnai G2 Spirit, Tecnai) and scanning electron microscopy (SEM, MIRA3 FEG-SEM, TESCAN). The SEM procedures were based on a previous study[28]. Generally, 100 μL of the exosome suspension was frozen overnight in the refrigerator and then transferred to a vacuum dryer for lyophilization. An appropriate amount of freezing glue was applied to the sample table and then the exosome lyophilized powder was spread onto the sample table. The samples were then coated with gold with an ion sputterer and observed under the microscope.

To assess the absolute size distribution of C2C12-Exos, they were analyzed using a NanoSight NS300 (Malvern, United Kingdom). The particles were automatically tracked and sized using nanoparticle tracking analysis (NTA) based on Brownian motion and diffusion coefficients.

Table 1 Primary antibodies used in the experiment

Antibody	Source	Catalog No.	Type	Dilution	M.W. (kD)
CD63	Affinity	AF5117	Rabbit mAb	1:1000(W.B.)	47
CD9	Affinity	AF5139	Rabbit mAb	1:2000(W.B.)	23
Alix	Affinity	ab275377AF0184	Rabbit mAb	1:2000(W.B.)	95
HSP60	Affinity	AF0199	Rabbit mAb	1:2000(W.B.)	60
iNOS	Affinity		Rabbit mAb	1:1000(W.B.)	130
ARG1	Affinity	DF6657	Rabbit mAb	1:1000(W.B.)	35
CD86	Abcam	ab220188	Rabbit mAb	1:1000(W.B.)	38
				1:100(IF)	
CD86 PE	eBioscience	12-0862-81	Rat mAb	0.125 μg/test	(Flow Cyt)
CD206	Affinity	DF4149	Rabbit mAb	1:1000(W.B.)	120
CD206	Abcam	ab64693	Rabbit mAb	1:500(IF)	
	eBioscience	17-1631-80	Rat mAb	0.25 μg/test	(Flow Cyt)
CD163APC	Abcam	ab51263	Mouse mAb	1:1000(W.B.)	227
MYHC				1:500(IF)	
MyoD1	Affinity	AF7733	Rabbit mAb	1:1000(W.B.)	60
MyoD1	Proteintech	18943-1-AP	Rabbit PAb	1:200(IF)	
MyoG	Abcam	ab1835	Mouse mAb	1:1000(W.B.)	25
Collage 1	Affinity	AF7001	Rabbit mAb	1:1000(W.B.)	140
α-SMA	Affinity	AF1032	Rabbit mAb	1:1000(W.B.)	42
Tubulin	Affinity	T0023	Mouse mAb	1:1000(W.B.)	55
GAPDH	Affinity	AF7021	Rabbit mAb	1:1000(W.B.)	37
F4/80 APC	eBioscience	47-4801-80	Rat mAb	0.125 μg/test	(Flow Cyt)

α-SMA: α-smooth muscle actin.

To identify the surface markers of C2C12-Exos, exosomes were assessed by flow cytometry analysis using a commercially Exo-Flow capture kit, including CD9, CD47, CD63, and CD81 flow antibodies (System Biosciences, CA, United States). These procedures were *per* the published studies[29].

Additionally, the C2C12-Exos were identified by Western blotting with anti-CD63, anti-CD9, anti-Alix, and anti-HSP60 antibodies (purchased from Abcam or Affinity) (Table 1).

Cell intervention

C2C12 and RAW264.7 cells were incubated at 1 × 10^7 cells in 10 cm plates and divided into groups according to different IF-C2C12-Exos concentrations or conditioned medium. After 24 or 48 h of incubation, macrophages were collected for flow cytometry/western blot or fixation to assess histological changes. After 48 h of treatment with high concentrations of IF-C2C12-Exos, macrophages were collected in conditioned medium. In this study, we defined this as M1CM, meaning that the macrophages in the medium were more of the M1 subtype rather than M2 or M0 macrophages. In addition, C2C12 cells were incubated with exosomes for 24 h for further experiments, including proliferation and differentiation (Figure 1).

Exosome labeling with PKH67

Isolated C2C12-Exos were labeled with PKH67 (a Green Fluorescent Labeling Kit (Sigma, Aldrich, MINI67-1KT)), and the procedures followed the manufacturer's protocol. C2C12-Exos or PBS were stained with PKH67 dye in 500 μL of Diluent C solution for 5 min at room temperature. After that, 1 mL 1% BSA was used to stop the labeling process. Then the re-purified exosomes underwent ultracentrifugation with a

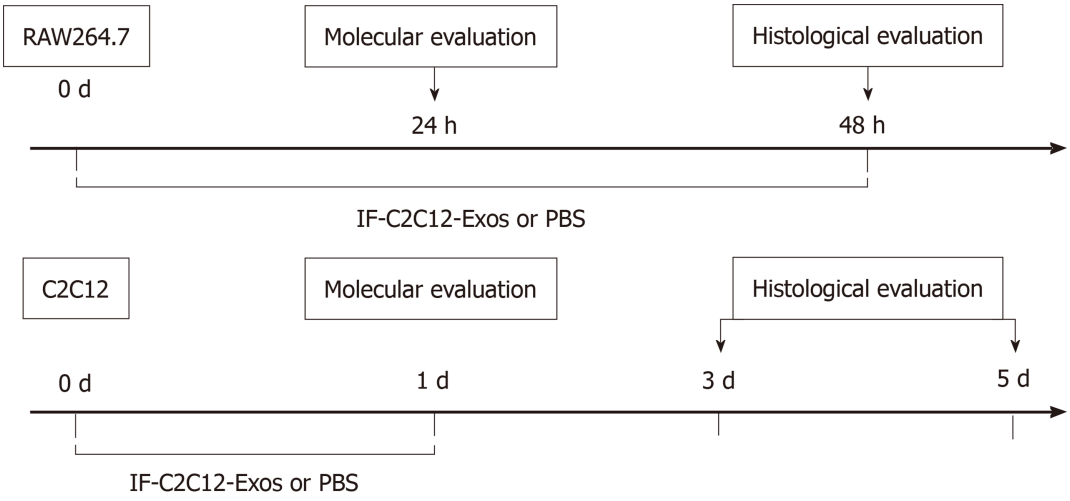

Figure 1 Experimental design for both the *in vitro* and *in vivo* studies. IF-C2C12-Exos, Myoblast-derived exosomes within the inflammatory environment; Molecular evaluation included Western Blot, ELISA, immunofluorescence, and flow cytometry. Histological evaluation included immunofluorescence and electron microscopy. PBS: Phosphate-buffered saline.

PBS rinse for 30 min. The labeled PBS or C2C12-Exos were co-incubated with C2C12 cells and macrophages (M0) for 12 h at 37°C, in a 5% CO_2 cell incubator. After incubation, the culture medium was discarded and the cells were washed with PBS three times. Cells were fixed with 4% PFA and nuclei were counterstained with DAPI for 5 min. The uptake of labeled exosomes by C2C12 and macrophages was observed by a fluorescence microscope (ECHO Revolve, United States). Six random images of cells were taken, and PKH-67 positive cells were counted. The total number of cells was calculated using the DAPI staining method. The positive rate of PKH-67 = PKH-67 positive cells/total number of cells.

Histological analysis

Macrophage differentiation and morphology: M0 macrophages were incubated with different concentrations of IF-C2C12-Exos for 2 d (1×10^9, 1×10^{10}, 2×10^{10}/medium). Macrophage cultures were then viewed directly under a microscope (ECHO Revolve, United States).

Immunofluorescence staining: To examine the expression and location of target proteins, cells were immunofluorescently stained as previously described[30]. The primary antibodies used were anti-CD86, anti-CD206, anti-MYHC, anti-iNOS, and anti-MyoD1. DAPI was used to locate the nuclei of the cells. Images were taken using fluorescence microscopy (ECHO Revolve, United States).

Giemsa stain: C2C12 cells were washed with PBS, fixed with 4% PFA for 10 min, and rinsed three times with fresh PBS for 5 min each time. The fixed cells were then incubated with Giemsa stain (Phygene, China) for 50 min. Cells were rinsed with tap water for 3 min and then photographed using an inverted microscope. Three images were taken randomly at 200 × magnification for each well. The numbers of total and fused cell nuclei were counted. Cell counting was performed using ImageJ. The fusion rate was defined as the percentage of total nuclei being in myotubes/total nuclei of C2C12 cells. The diameter and length of the myotube were analyzed by Image-Pro Plus 7.0.

Flow cytometry for M1/M2

Flow cytometry was performed using anti-CD86-PE, anti-CD163-APC, and anti-F4/80-FITC (Thermo/eBio). The percentage of CD86 +/CD163 +/F4/80 + cell population (macrophages) was evaluated using Cytomics™ FC 500 (Beckman Coulter). In detail, different groups of macrophages were collected with flow cytometry staining buffer (eBioscience). Then, 2 μL antibody was added to each 100 μL of cell suspension for 60 min at 4°C in the dark. Then, 5 mL staining buffer was put into each tube and the cell suspension was centrifuged for 5 min (500 ×g, 4°C). The washing procedures were repeated three times. Finally, the cell precipitation was re-suspended in 200 μL PBS for flow cytometry analysis.

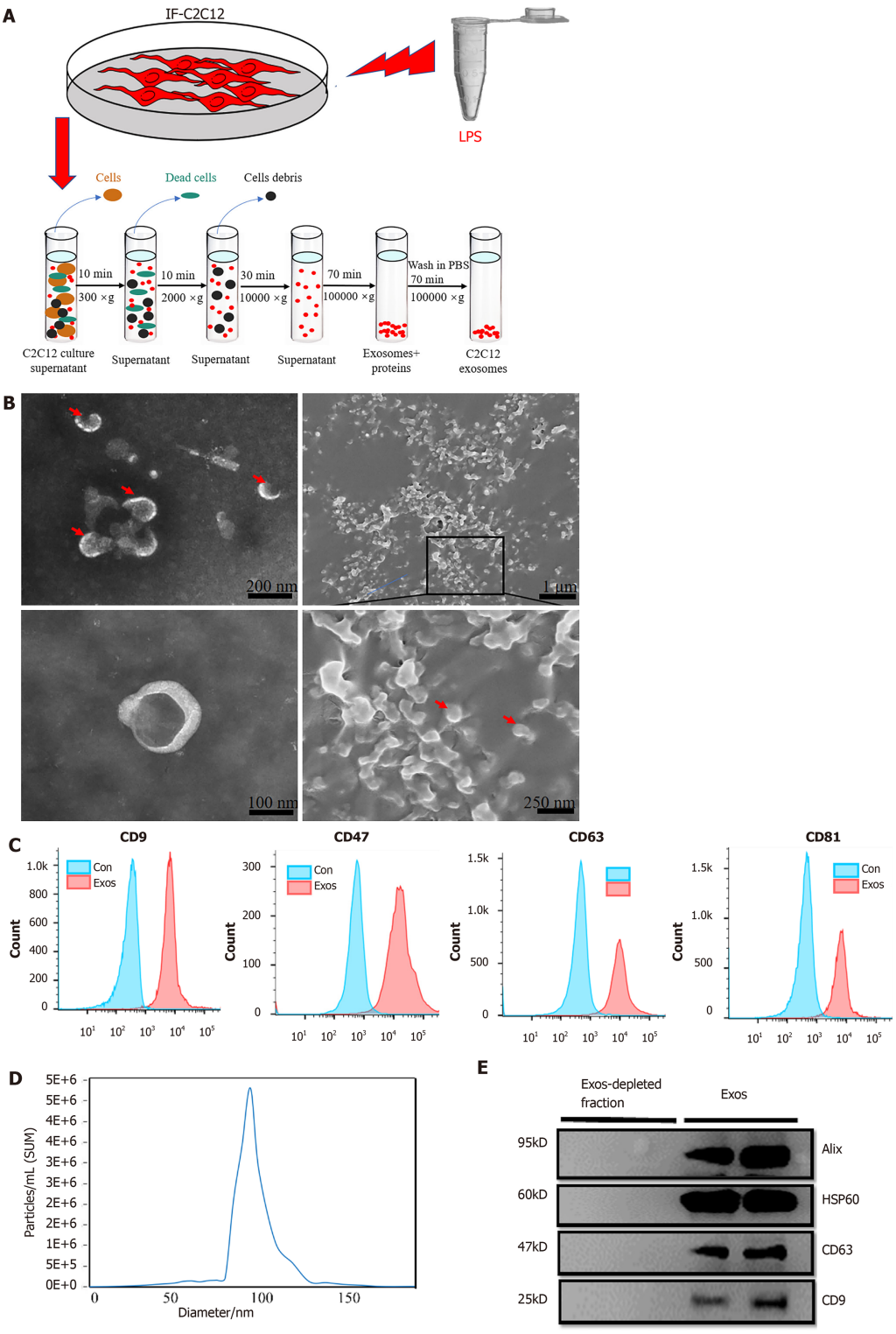

Figure 2 Purification, isolation, and characterization of C2C12-Exos. A: Flowchart of C2C12-Exos purification based on differential ultra-centrifugation. Lipopolysaccharide, 1000 ng/mL for 1 d; B: The morphology of C2C12-Exos was observed by transmission electron microscopy (left) and scanning electron microscopy (right). The red arrows indicate representative exosomes; C: Representative flow cytometry plots showing the phenotypes of exosome markers, including CD9, CD47, CD63, and CD81; D: The particle size distribution of C2C12-Exos was analyzed by the qNano platform; E: Western blotting showed the presence of exosomal markers, including CD63, HSP60, Alix, and CD9. The four lanes represent different exosomal proteins and deproteinized supernatants extracted from the two independent conditioned media. LPS: Lipopolysaccharide.

Western blot

Protein was extracted and analyzed using an established method[31]. Briefly, total protein from C2C12 was collected using RIPA lysis buffer (R0010; Solarbio, Beijing, China) with phenylmethanesulfonyl fluoride (PMSF; Solarbio, Beijing, China). Protein concentrations were measured using a BCA Protein Assay Kit (Beyotime Biotech-

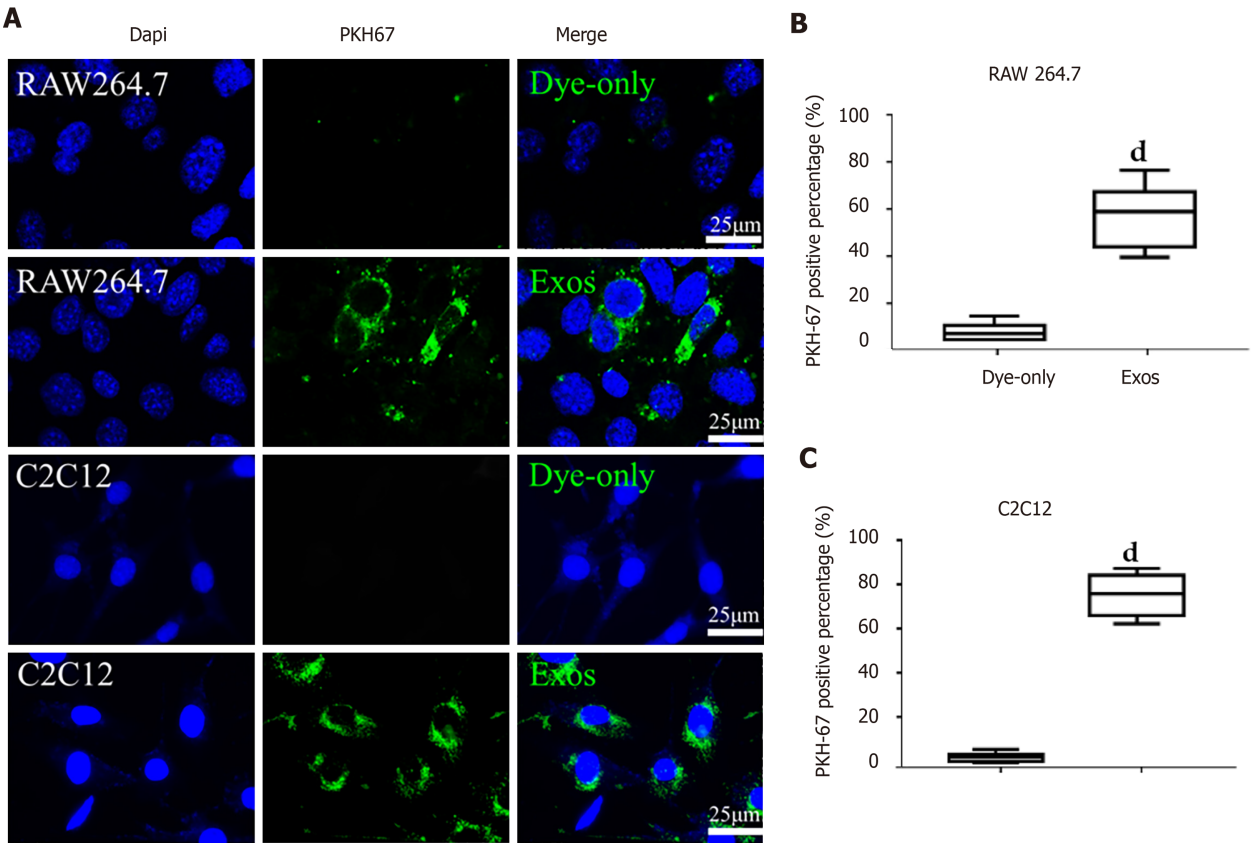

Figure 3 C2C12-Exos were taken up by C2C12 and RAW264.7 cells *in vitro*. A: Representative images of the uptake of PKH67-labeled exosomes (green) by RAW264.7 cells or C2C12 cells (DAPI blue) and fluorescence uptake by negative control samples. Dye-only, only PKH67 incubated with RAW264.7 cells and C2C12 cells; scale bar = 25 μm; B and C: The percentage of PKH67 positive cells in RAW264.7 or C2C12 is presented. $^{d}P < 0.0001$, $n = 6$.

nology, Shanghai, China). Next, 10 μg protein samples from each group were separated by 10% SDS-PAGE. Afterward, they were transferred to nitrocellulose membranes. 5% non-fat milk dissolved in Tris-buffered saline containing Tween-20 was utilized to block the blots before applying primary antibodies overnight at 4°C. Anti-CD206, anti-Arginase 1, anti-iNOS, anti-CD86, anti-MYHC, anti-MyoD1, anti-MyoG, anti-Col 1, anti-α-SM, anti-β-Tubulin, and anti-GAPDH antibodies were applied as primary antibodies (Table 1). Each group contained 4 protein samples for calculation ($n = 4$).

C2C12 proliferation and migration

BrdU test: The proliferation of C2C12 cells was determined using the 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay kit (Cell Signaling Technology, MA, United States) according to the manufacturer's instructions. Briefly, cells were seeded into a 6-well plate with 1×10^6 cells *per* well. BrdU solution was added into each well 3 h prior to IF-C2C12-Exos treatment. Cell proliferation was evaluated using the average number of BrdU + cells *per* view.

Scratch assay: To assess the cell migration properties of C2C12, an *in vitro* scratch assay was performed by scratching a straight line in the middle of cells cultured for 24 h. Then, different concentrations of IF-C2C12-Exos were applied for 24 h and observed under an inverted microscope. Cell migration ability was evaluated by the percentage of wound-healing rate (distance migrated/ original wound distance × 100%).

CCK-8 assay: The Cell Counting Kit-8 (CCK-8, Beyotime Biotechnology, Shanghai, China) assay was used to assess the viability of C2C12 cells after IF-C2C12-Exos treatment. The general procedure followed that outlined in a previous study[32]. Briefly, primary cells were seeded into a 96-well plate (Thermo Fisher Scientific, MA, United States) with 1×10^3 cells *per* well and treated with IF-C2C12-Exos for 24 h. Next, CCK-8 solution (10 μL) was applied to each well, and the plate was incubated for 2 h and the absorbance of each well was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, United States). Cell viability was evaluated by average absorbance of the IF-C2C12-Exos treatment group/absorbance of control group ×

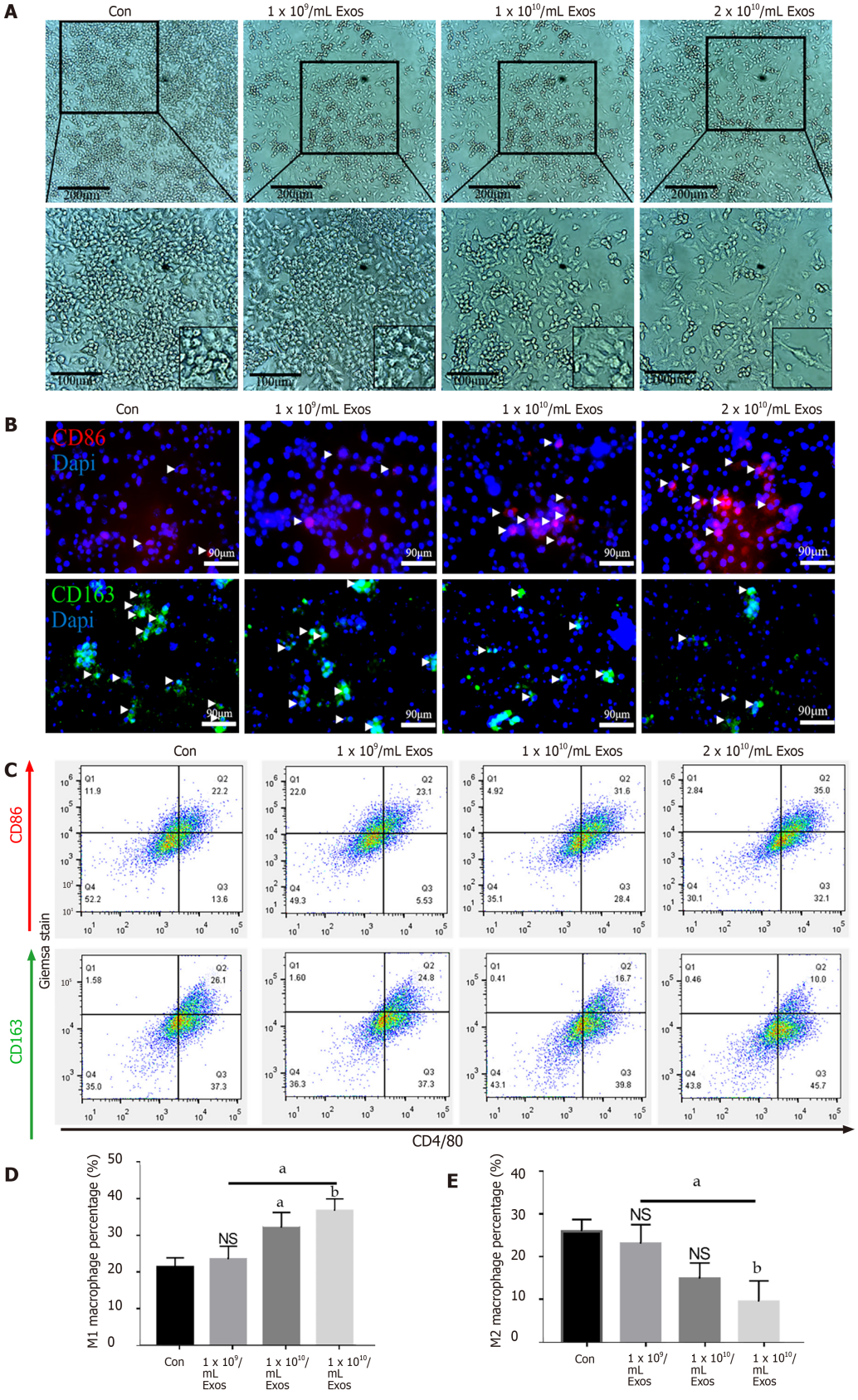

Figure 4 IF-C2C12-Exos induced M1 macrophage polarization *in vitro*. A: Images of macrophages cultured with different concentrations of C2C12-Exos for 2 d (1 × 10^9, 1 × 10^{10}, 2 × 10^{10}/medium). Scale bar = 200 μm or 100 μm; B: Immunofluorescence localization of CD86 (red), marker of M1 and CD206 (green), marker of M2 after culture with different concentrations of C2C12-Exos for 2 d. Scale bar = 90 μm. Arrows indicate CD206 or CD86 positive cells; C: Representative flow cytometry plots showing the percentages of M1 (CD86 + CD4/80+) and M2 (CD163 + CD4/80+) phenotype in macrophages after culture with different concentrations of C2C12-Exos for 24 h; D: Quantification of flow cytometry data (n = 3); E: M2 macrophages percentage. Data are presented as mean ± SD. ^aP < 0.05; ^bP < 0.01. NS: Not significant.

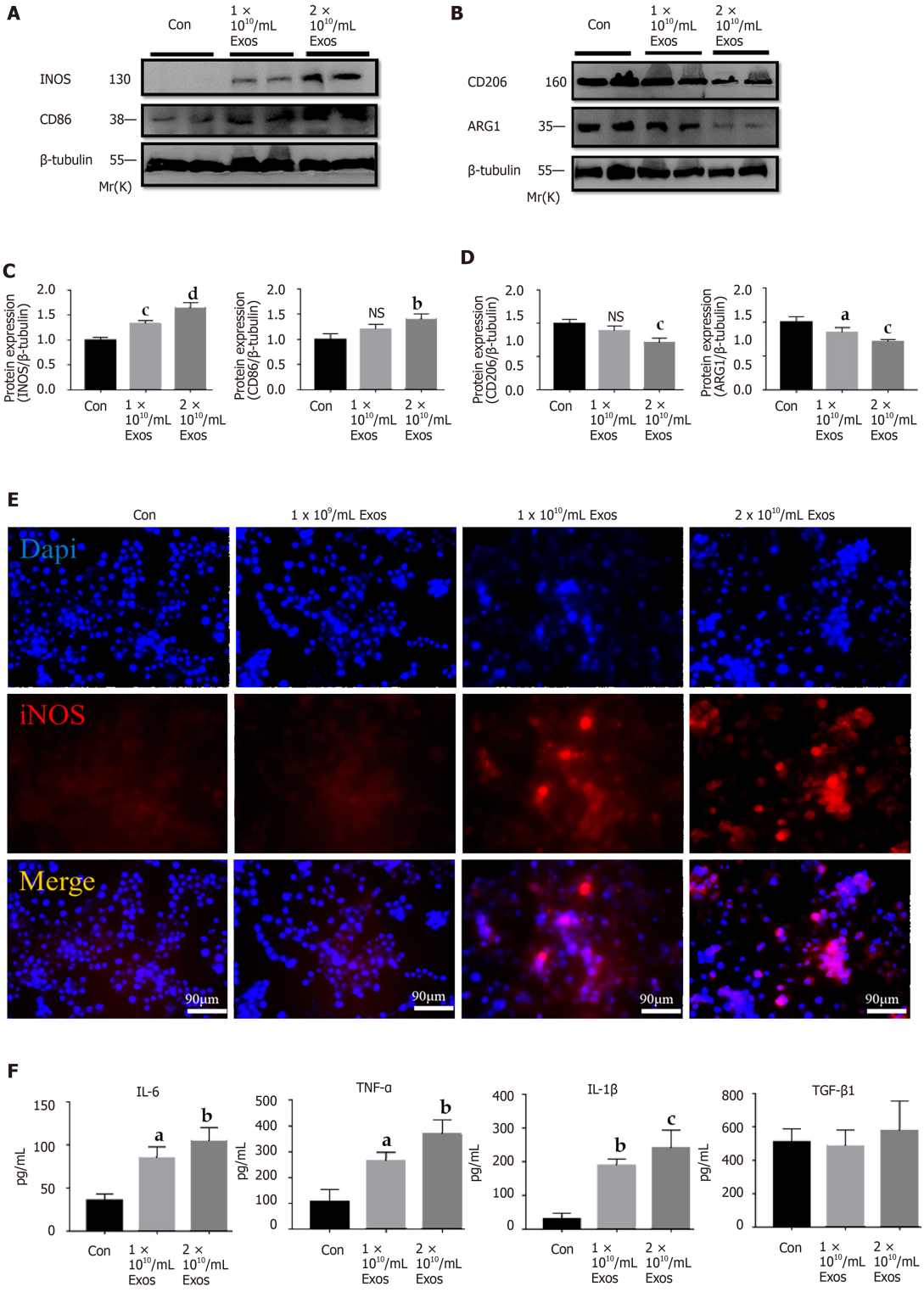

Figure 5 IF-C2C12-Exos induced inflammatory reactions of macrophages *in vitro*. A-D: iNOS, CD86, CD206, and Arg1 protein levels in macrophages were determined by western blot after culturing with different concentrations of C2C12-Exos for 24 h (1 × 10^{10}, 2 × 10^{10}/medium). (n = 4). Data are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$; E: Immunofluorescence localization and relative expression of iNOS, a marker of inflammatory level, in macrophage medium after culture with different concentrations of C2C12-Exos for 24 h. Scale bar = 90 μm; F: The concentration of cytokine interleukin (IL)-6, transforming growth factor-β, tumor necrosis factor-α, and IL-1β in supernatants of macrophage cells after culture with IF-C2C12-Exos or phosphate-buffered saline for 24 h measured by ELISA (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. PBS: Phosphate-buffered saline. IL: Interleukin; TGF-β: Transforming growth factor-β; TNF-α: Tumor necrosis factor-α; NS: Not significant.

100%.

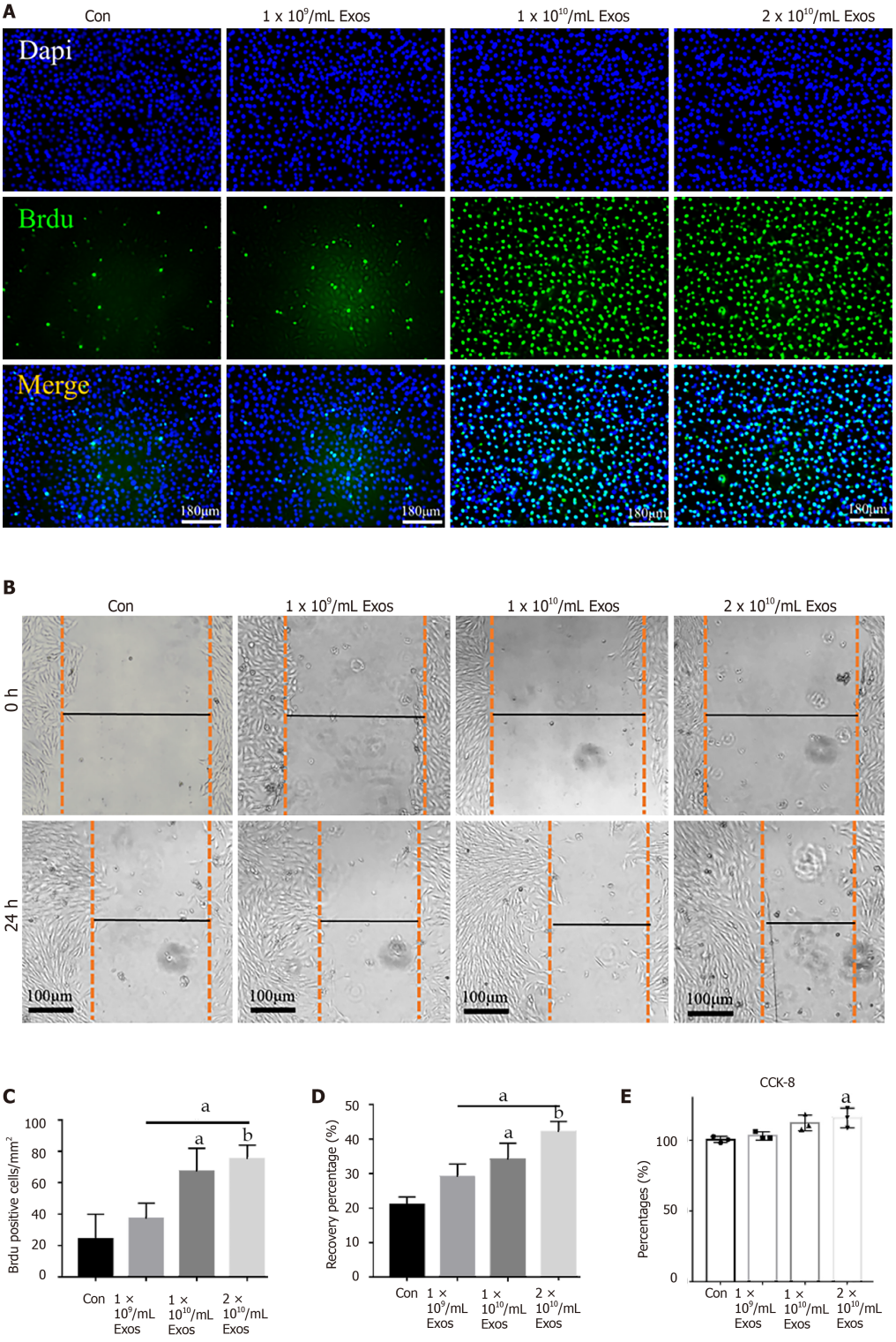

Figure 6 IF-C2C12-Exos stimulated C2C12 proliferation *in vitro*. A: Cell proliferation ability of C2C12 was determined using the BrdU incorporation assay after 24 h IF-C2C12-Exos incubation. Green signal = BrdU. Scale bar = 180 μm; B: The cell migration ability of C2C12 was determined by the wound-healing assay after 24 h IF-C2C12-Exos incubation; C and D: Quantification of BrdU and wound-healing data (n = 3). Data are presented as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$; E: Cell proliferation ability of C2C12 was further determined using the CCK-8 assay. Data are presented as mean ± SD. $^{a}P < 0.05$.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits, including interleukin (IL)-6, IL-1β, TGF-β, and tumor necrosis factor-α (TNF-α), were purchased from Laizee (LEM060-2, LEM012-2, LEM822-2, LEM810-2). The protocol followed previous studies[33]. Cell supernatants were collected from each group and the kits were then utilized following the manufacturer's instructions. In detail, 100 μL of samples and standard samples were added to the corresponding well, the plates were sealed and incubated at room

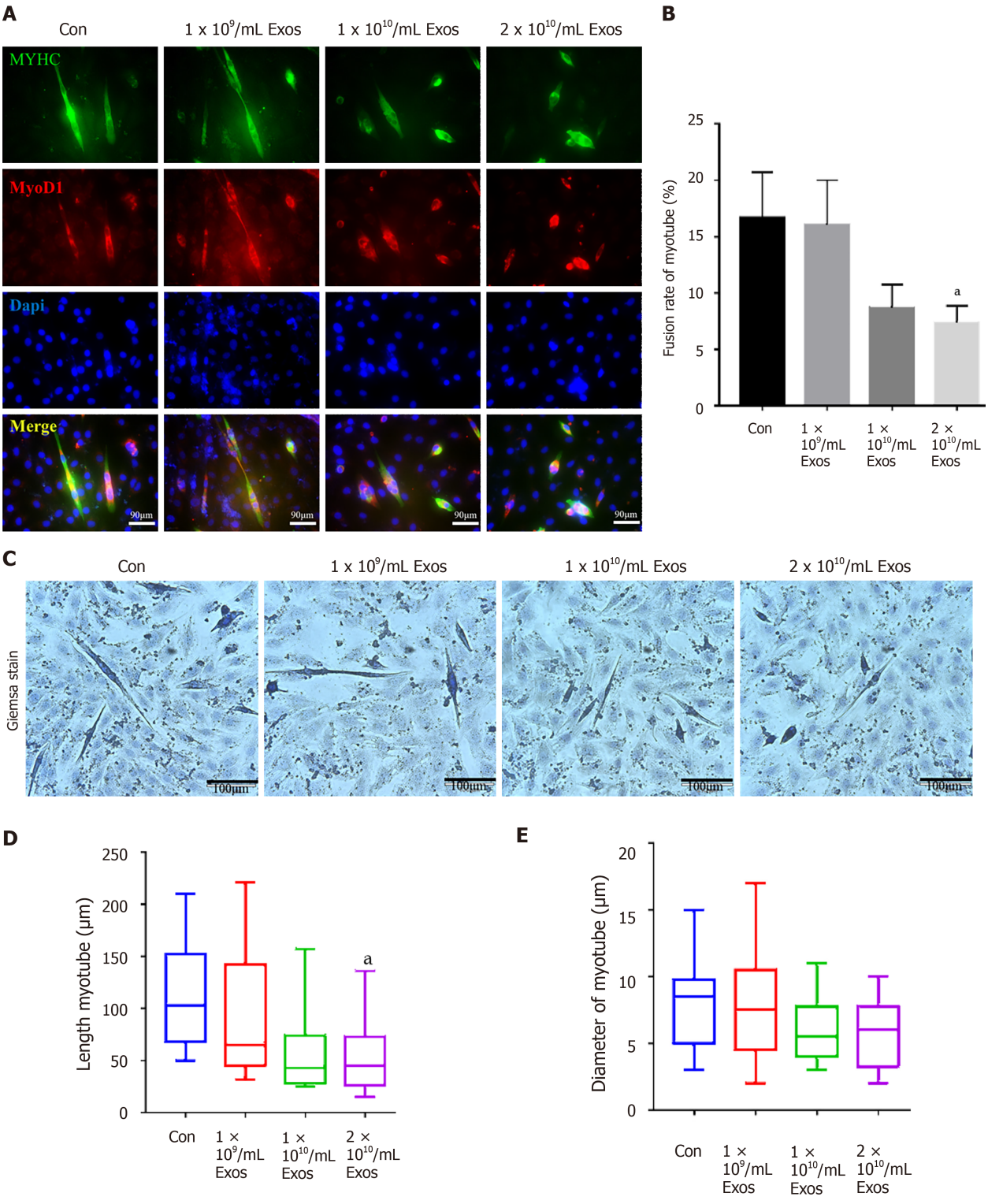

Figure 7 IF-C2C12-Exos impaired early C2C12 muscle differentiation *in vitro*. A: Immunofluorescence was used to detect relative expression and distribution of MYHC and Myod1 on day 2 after different treatments. Green, red, and blue signals represent MYHC, Myod1, and nucleus, respectively. Scale bar = 90 μm; B, D and E: Quantification of myotube length, diameter, and fusion rate. Data are presented as mean ± SD. n = 12 or 3. $^{a}P < 0.05$; C: Representative images of myotube after culture with different concentrations of IF-C2C12-Exosomes for 24 h by Giemsa staining (2 d 2% horse-serum incubation). Scale bar = 100 μm.

temperature for 2 h. The samples were then discarded and the plates were washed 5 times with 300 μL of wash and drained on paper. Then 100 μL avidin HRP solution was added to each well, the plate was sealed, and incubated at room temperature for 30 min. The washing procedure was repeated. Next, 100 μL TMB solution was added to each well, the plate was sealed, and incubated for 15 min at room temperature. Finally, 50 μL of terminator solution was added to terminate the reaction and the plate was analyzed at 450 nm.

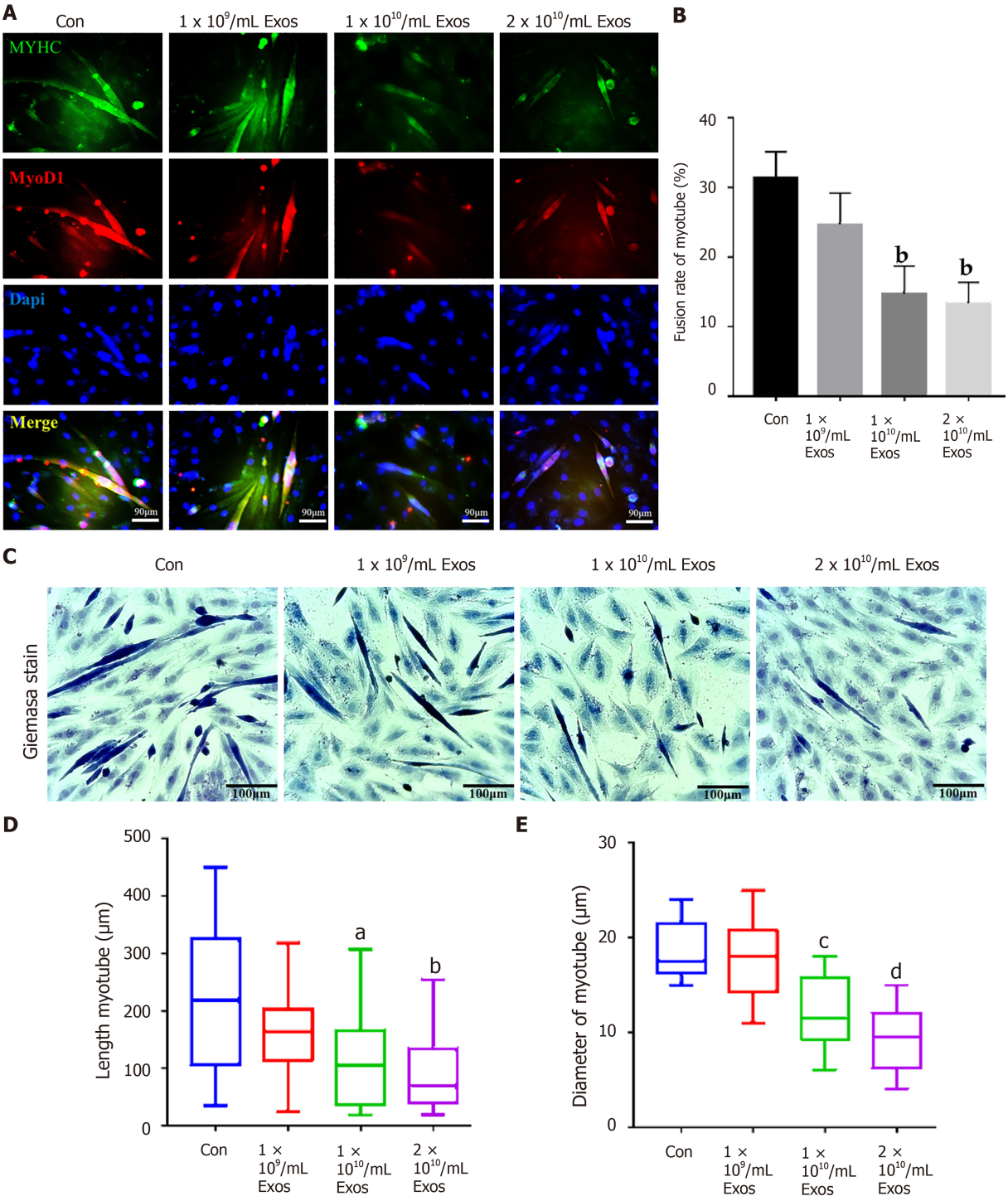

Figure 8 IF-C2C12 Exos impaired C2C12 muscle differentiation *in vitro*. A: Immunofluorescence was used to detect relative expression and distribution of MYHC and Myod1 on day 4 after different treatments. Green, red, and blue signals represent MYHC, Myod1, and nucleus, respectively. Scale bar = 90 μm; B, D and E: Quantification of myotube length, diameter, and fusion rate. Data are presented as mean ± SD. n = 12 or 3. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$; C: Representative images of myotube after culture with different concentrations of IF-C2C12-Exosomes for 24 h by Giemsa staining (4 d 2% horse-serum incubation). Scale bar = 100 μmT.

Statistical analysis

All experiments were performed at least three times. Data were analyzed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, United States) and presented as the mean ± SD. Significance was typically analyzed by Student's t-test, or one-way ANOVA followed by post hoc LSD test. $P < 0.05$ was regarded as significant. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ and $^{d}P < 0.0001$.

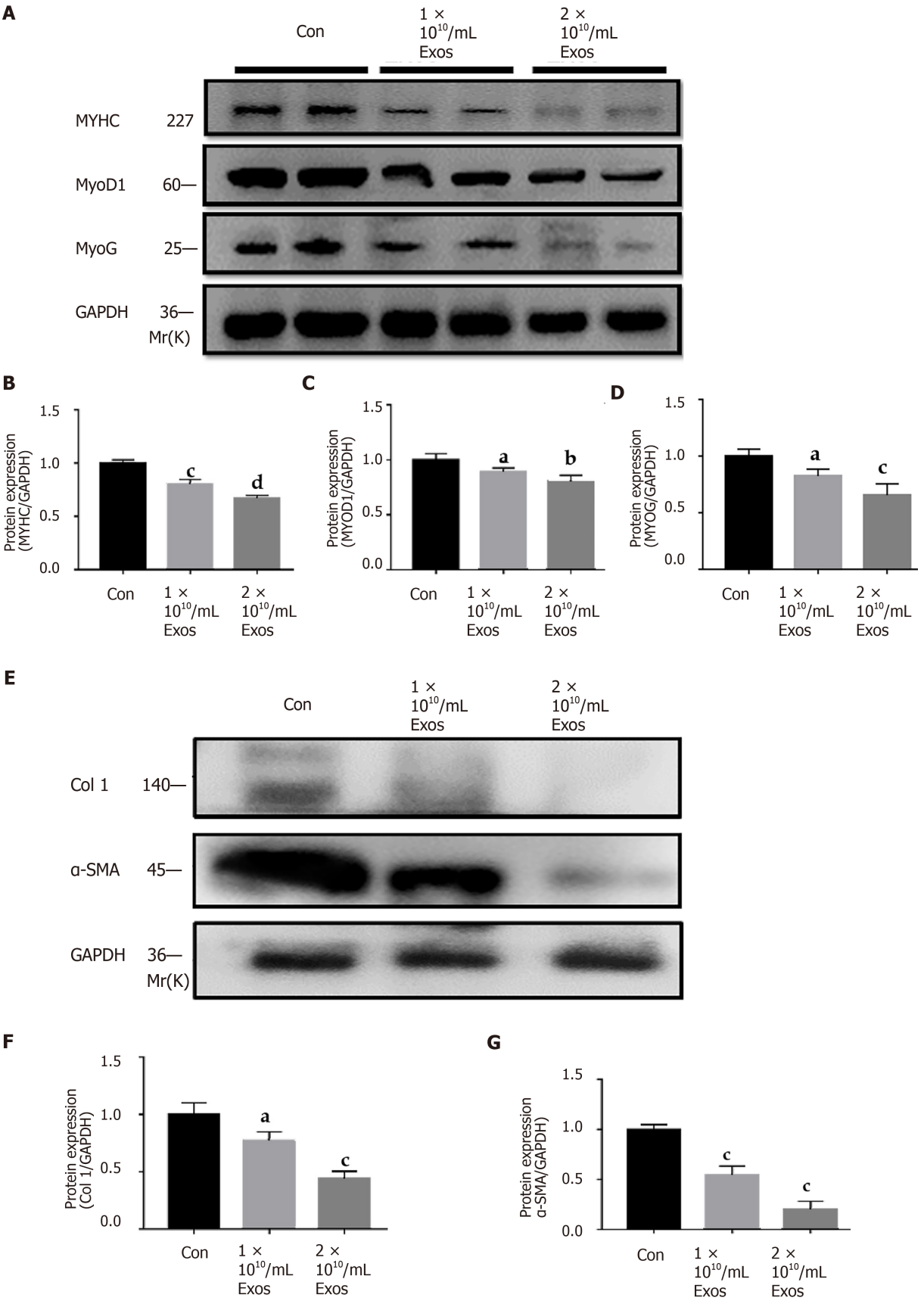

Figure 9 IF-C2C12 Exos down-regulated C2C12 fibrogenic/myogenic differentiation-related proteins *in vitro*. A-D: MYHC, MYOD1, and MYOG protein levels in C2C12 medium were determined by western blot after incubation with different concentrations of C2C12-Exos for 24 h (1×10^9, 1×10^{10}, 2×10^{10} /medium) ($n = 4$); E-G: Col 1 and α-smooth muscle actin. Data are presented as mean ± SD. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$, $^dP < 0.0001$. α-SMA: α-smooth muscle actin.

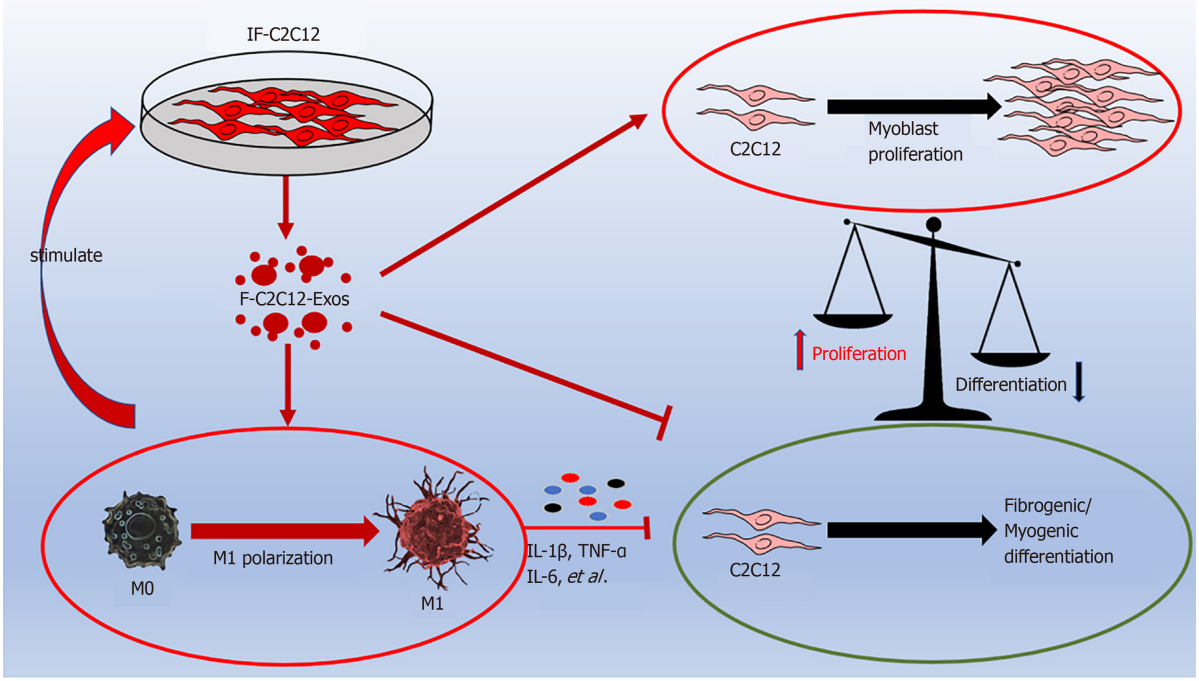

Figure 10 Graphic abstract. IF-C2C12-Exos promoted M1 macrophage polarization and myoblast proliferation, and inhibited myoblast fibrogenic/myogenic differentiation. IL: Interleukin; TNF-α: Tumor necrosis factor-α.

C2C12-CM regulated macrophage polarization

We first investigated the effects of conditioned medium on macrophage polarization in normal myocytes or myoblasts in an inflammatory environment. LPS at 500 ng/mL and IL-4 at 20 ng/mL were utilized as a positive control, while the fresh exosome-depleted medium served as a negative control. We found that IF-CM and LPS induced M1 polarization (significantly more CD86+ cells compared with the control group), while NC-CM and IL-4 promoted M2 polarization (markedly more CD163+ cells compared with the control group) (Supplementary Figure 1). These results suggested that there was crosstalk between myoblasts and macrophages through their secretion. Therefore, we performed further experiments.

M1CM hindered myoblast myogenic differentiation

IF-C2C12 stimulated macrophages towards the M1 subtype for two days. Fresh medium was then added to the macrophages and 24 h later conditioned medium was collected to treat normal C2C12 (Supplementary Figure 2A). Giemsa stain and immunofluorescence results showed that the myotubes after M1CM administration were smaller and fewer than those of controls, which suggested that the myoblast myogenic differentiation ability was weakened by M1CM (Supplementary Figure 2B and C).

Identification and characterization of C2C12-Exos after LPS stimulation

Under transmission and SEM, C2C12-Exos appeared spherical or globular in shape (Figure 2A and B). Flow cytometry results demonstrated that exosomal markers (CD9, CD47, CD63, and CD81) were highly expressed in C2C12-Exos (Figure 2C). The NTA experiment evaluated the size of C2C12-Exos. The diameters ranged from 50-130 nm, which are consistent with the data from previous studies (Figure 2D). Western blot results showed that exosomal marker proteins (CD9, CD63, Alix, and HSP60) were highly expressed in C2C12-Exos, while these proteins were expressed in the exosome-depleted fractions (Figure 2E).

Exosomes tracing in vitro

To investigate how C2C12-Exos communicated with C2C12 and macrophages, C2C12-Exos were added to cultured C2C12 and RAW264.7 cells. PKH-67 (green) labeled C2C12-Exos were internalized into C2C12/macrophages and were identified in the cytoplasm after 12 h of co-culture. The Dye-only group showed no PKH67 signals (Figure 3A). The PKH-67 positivity rate was significantly higher in macrophages and C2C12 than in the Dye-only group (Figure 3B and C).

IF-C2C12-Exos induced M1 macrophage polarization in vitro

To examine the effects of IF-C2C12-Exos on macrophages, different concentrations of IF-C2C12-Exos were added to the culture system. After 48 h, as the concentration increased, the macrophages became more elongated and predominantly spindle-shaped compared with their original circular shape (Figure 4A). In addition, immunofluorescence staining showed an increase in CD86-positive cells and a decrease in CD206-positive cells as the concentration of IF-C2C12-Exos increased (Figure 4B). Moreover, flow cytometry results showed a significant increase in the proportion of M1 macrophages and a significant decrease in the proportion of M2 macrophages in the IF-C2C12-Exos group (Figure 4C-E). These data demonstrate that IF-C2C12-Exos induce the polarization of macrophages towards M1 *in vitro*.

IF-C2C12-Exos induced inflammatory reactions of macrophages in vitro

To further investigate the effects of IF-C2C12-Exos on macrophages, inflammatory-related factors were evaluated. The results of western blot demonstrated that IF-C2C12-Exos significantly up-regulated the protein expression (iNOS and CD86) and down-regulated that of CD206 and Arg1 (Figure 5A-D). In addition, immunofluorescence staining results showed that the relative expression and area of iNOS gradually increased with increasing concentrations of IF-C2C12-Exos (Figure 5E). Furthermore, ELISA results showed that the concentrations of cytokine IL-6, TNF-α, and IL-1β in the culture supernatants significantly increased, while that of TGF-β did not show obvious changes after IF-C2C12-Exos treatment for 24 h (Figure 5F). Taken together, these data suggested that IF-C2C12-Exos can induce inflammatory reactions of macrophages *in vitro*.

IF-C2C12-Exos promoted C2C12 proliferation in vitro

To evaluate the effects of IF-C2C12-Exos on normal myoblasts, we first examined the proliferative capacity of the cells. The BrdU assay results showed that treatment with high concentrations of IF-C2C12-Exos (1×10^{10}, 2×10^{10}/medium) significantly increased the mean number of BrdU-positive cells, indicating that these C2C12 cells gained higher proliferation capacity (Figure 6A and C). Next, the wound-healing rate in the IF-C2C12-Exos group was significantly higher than that of the control group, which suggested that the migration capacity of C2C12 was enhanced by the IF-C2C12-Exos (Figure 6B and D). Furthermore, the results of the CCK-8 assay revealed a significant increase in the viability of C2C12 cells in the 2×10^{10}/medium concentration group after IF-C2C12-Exos treatment, while the low concentration group (1×10^{9}, 1×10^{10}) showed a small but insignificant increase in viability (Figure 6E). Overall, these data demonstrate that IF-C2C12-Exos can stimulate normal C2C12 proliferation *in vitro*.

IF-C2C12-Exos impaired C2C12 differentiation in vitro

The fusion index of control myoblasts in differentiation and those treated with IF-C2C12-Exos were slightly different at the early stages (2 d) under all conditions tested (Figure 7A and B). More interestingly, after 4 d of differentiation, the fusion indices of the IF-C2C12-Exos treatment groups were significantly lower than that of the control group (14%-16% *vs* 32%) (Figure 8A and B).

In addition, our data also showed that myotube diameter and length were significantly affected by IF-C2C12-Exos treatments, with IF-C2C12-Exos inducing a significant decrease in myotube size at both early and late stages of muscle differentiation. As a result, there was a significant difference in the diameter and length of control and IF-C2C12-Exos myotubes, and this difference increased at higher IF-C2C12-Exos concentrations (Figures 7C-E and 8C-E). Finally, modulation of the myoblast fibrogenic/myogenic differentiation process in response to IF-C2C12-Exos treatment was investigated by western blot analysis. MyoG, MyoD1, MYHC, α-smooth muscle actin (SMA), and Col 1 protein expression levels showed a significant downregulation after 24 h of IF-C2C12-Exos treatment (Figure 9). Altogether, these data suggest that IF-C2C12-Exos impairs C2C12 muscle differentiation *in vitro* and triggers a shift in the balance of proliferation/differentiation towards proliferation.

DISCUSSION

This is the first study to demonstrate that myoblasts within the inflammatory environment crosstalk with surrounding macrophages. In this study, IF-C2C12-Exos

was shown to promote M1 polarization of M0 macrophages and to influence the balance of myoblast proliferation/differentiation *in vitro*.

Following acute mechanical injury, skeletal muscle develops significant inflammation[34,35]. If the M1 stage of macrophages persists after acute muscle injury, it will result in a prolonged inflammatory environment in the damaged area[14,36]. However, the underlying mechanism by which the M1 phenotype remains at the early and mid-stage of injury is less well understood. The similar appearance time of myoblasts and M1 macrophages after skeletal muscle injury may suggest a crosstalk between themselves[12,16]. CD86 and CD163 expression levels can reflect the polarization stage of macrophages[37]. In this study, the exosomes from inflammatory C2C12 myoblasts was found to induce higher levels of CD86 expression (M1 marker) than that of CD163 expression (M2 marker) in macrophages. Furthermore, we found that the inflammatory reactions in macrophages were also aggravated by IF-C2C12-Exos. A previous study[23] reported that exosomes from H_2O_2 treated myotubes could stimulate RAW264.7 macrophages to express higher levels of IL-6, which is consistent with our findings. However, we found other inflammatory factors, such as IL-1β, TNF-α, and iNOS, were also upregulated in macrophage cultures following administration of high concentrations of IF-C2C12-Exos. This suggests that IF-C2C12-Exos may regulate macrophages to exert higher level inflammatory responses than those of H_2O_2-myotube-Exos. Interestingly, IF-C2C12-Exos reduced M2 macrophage expression, while IF-C2C12-CM did not exert that effect but also induced M1 polarization. Many studies have proved that macrophage polarization was regulated by surrounding environmental factors including cytokines and exosomes[38,39]. These results suggested that IF-C2C12-Exos can regulate the polarization of macrophages and maintain a prolonged inflammatory environment, while myoblasts in the inflammatory environment can continue to secrete IF-C2C12-Exos. This local positive feedback intercellular mechanism was observed in Xu *et al*[20]'s study, whereby C2C12-Exos promoted pre-osteoblasts differentiation to osteoblasts[20].

In this study, IF-C2C12-Exos were found to impair C2C12 differentiation and promote proliferation *in vitro*. In detail, decreased levels of MyoD, MyoG, and MYHC protein levels[25,26,40] suggesting that IF-C2C12-Exos reduced the myogenic differentiation ability of myoblasts. Induction experiments of myogenic differentiation also provided direct evidence for this result. In addition, the BrdU, CCK-8, and scratch assays[41] showed that a higher ability of myoblast proliferation was induced by IF-C2C12-Exos. This result is consistent with previous literature where exosomes from C2C12 myotube after H_2O_2 or TNF-α/interferon-γ treatment enhanced proliferation but impaired myogenic differentiation[23,42].

Significant and prolonged inflammation after acute muscle injury can result in muscle fibrosis[10,11,14,36]. Additionally, promoting M1 macrophages to M2 during the inflammatory phase after muscle injury prevents muscle fibrosis[27,43,44]. Interestingly, myocyte IF-C2C12-Exos treatment resulted in a significant decrease in protein levels of the fibrosis markers (Col 1, α-SMA), implying that the fibrogenic capacity of normal myoblasts was also suppressed[45]. We speculate that IF-C2C12-Exos are only secreted by myoblasts in the acute inflammatory stage (1-5 d after injury). They may disappear after day 5 due to inflammatory dissipation, meanwhile, M2 macrophages secrete a large amount of TGF-β, which activates myoblasts into fibroblasts, leading to ECM production[24,26,46]. However, due to prolonged inflammation caused by M1 macrophages, M2 polarization is incomplete and tissue remodeling is maladaptive, which leads to subsequent fibrosis[27,47]. Our results suggest that myoblasts passed information to surrounding myoblasts, telling them to grow faster but not differentiate under inflammatory conditions. This effect would have favored muscle regeneration, as large numbers of myoblasts are required to support muscle repair[48,49]. However, abnormal proliferation with down-regulated myogenic differentiation indeed hinders the muscle healing process after the acute inflammatory stage[50,51]. Additionally, the main effects of IF-C2C12-Exos are not to induce myoblast-derived fibrosis but to induce proliferation. A previous study proved that myoblasts are key in muscle fibrosis[52], and another study found that only promotion of myoblast proliferation cannot prevent fibrosis[6]. Therefore, we speculate that the IF-C2C12-Exos actually prepare the conditions for later fibrotic differentiation (accumulation of undifferentiated myogenic cells).

Together, the above results suggest that (1) IF-C2C12-Exos can induce macrophages towards M1 polarization, leading to a prolonged and aggravated inflammatory environment. In turn, the inflammatory factors stimulate myoblasts to produce more IF-C2C12-Exos, which forms a vicious circle; and (2) IF-C2C12-Exos can impair fibrogenic/myogenic differentiation, and lead to proliferation (Figure 10).

There are several limitations in this study that should be noted. First, we only utilized an *in vitro* model to study the effects of IF-C2C12-Exos on macrophages and myoblasts. However, muscle injury is a complex physiological and pathological issue with complex *in vivo* factors[53]. Further studies should consider validating the role of exosomes in animal models. Second, we only added IF-C2C12-Exos once; thus, multiple administrations should be considered, and the optimal concentration of exosomes should be studied in future research. Third, our study did not involve an in-depth mechanistic study. For example, myotubes were identified to promote myoblast fusion through exosomal miRNA[54]. Therefore, understanding the potential underlying mechanisms of how IF-C2C12-Exos can control macrophage polarization and influence the balance of myoblast proliferation/differentiation would be our future goal.

Given the pathophysiological significance of the findings of this study, further studies are needed to elucidate mechanisms responsible for these effects which deserve investigation. It is hoped that further studies will identify specific targets involved in muscle regeneration and fibrosis, such as lncRNAs and miRNAs.

CONCLUSION

Overall, this study demonstrates that IF-C2C12-Exos can promote M1 polarization and impair myoblasts differentiation. Normal or inflammatory myoblasts play a pivotal role, in fact, they release distinct Exos carrying a complex range of signals directed to the surrounding cells. Signals associated with Exos, by virtue of their diversity and specificity, may contribute to a fine reprogramming of the muscle regeneration process in a cooperative manner.

ARTICLE HIGHLIGHTS

Research background

More than half of sports injuries in athletes have been reported to be related to muscle damage. Severely injured muscles are prone to re-injury due to fibrotic scar formation caused by prolonged inflammation. How to regulate inflammation and suppress fibrosis is the focus of promoting muscle healing.

Research motivation

Recent studies have found that myoblasts and macrophages play important roles in the inflammatory phase following muscle injury; however, the crosstalk between these two types of cells in the inflammatory environment, particularly the exosome-related mechanisms, has not been well studied.

Research objectives

This study aimed to evaluate the effects of exosomes from inflammatory C2C12 myoblasts (IF-C2C12-Exos) on macrophage polarization and myoblast proliferation/differentiation.

Research methods

A model of inflammation was established *in vitro* by lipopolysaccharide stimulation of myoblasts. Multiple methods were used to isolate and identify the exosomes. Gradient concentrations of IF-C2C12-Exos were added to normal macrophages and myoblasts. PKH67 fluorescence tracing, microscopic morphology, Giemsa staining, immunofluorescence, ELISA assays, flow cytometry, western blot, BrdU test, scratch assay, and CCK-8 assay were conducted to determine the mechanism of IF-C2C12-Exos.

Research results

We found that the PKH-67-marked C2C12-Exos can be endocytosed by both macrophages and myoblasts. IF-C2C12-Exos induced M1 macrophage polari-zation and suppressed the M2 phenotype *in vitro*. These exosomes also stimulated the inflammatory reactions of macrophages. Furthermore, we demonstrated that IF-C2C12-Exos disrupted the balance of myoblast proliferation/differentiation, leading to enhanced proliferation and suppressed fibrogenic/myogenic differentiation.

Research conclusions

IF-C2C12-Exos can induce M1 polarization, resulting in a sustained and aggravated inflammatory environment that impairs myoblast differentiation, and leads to enhanced myogenic proliferation. These results demonstrate why prolonged inflammation occurs after acute muscle injury and provide a new target for the regulation of muscle regeneration.

Research perspectives

Given the pathophysiological significance of the findings of this study, further studies are needed to elucidate the mechanisms responsible for these effects which deserve investigation. It is hoped that further studies will identify specific targets involved in muscle regeneration and fibrosis, such as lncRNAs and miRNAs.

ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

Basic Study

ARPE-19 conditioned medium promotes neural differentiation of adipose-derived mesenchymal stem cells

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Abstract

BACKGROUND

Adipose-derived stem cells (ASCs) have been increasingly explored for cell-based medicine because of their numerous advantages in terms of easy availability, high proliferation rate, multipotent differentiation ability and low immunogenicity. In this respect, they have been widely investigated in the last two decades to develop therapeutic strategies for a variety of human pathologies including eye disease. In ocular diseases involving the retina, various cell types may be affected, such as Müller cells, astrocytes, photoreceptors and retinal pigment epithelium (RPE), which plays a fundamental role in the homeostasis of retinal tissue, by secreting a variety of growth factors that support retinal cells.

AIM

To test ASC neural differentiation using conditioned medium (CM) from an RPE cell line (ARPE-19).

METHODS

ASCs were isolated from adipose tissue, harvested from the subcutaneous region of healthy donors undergoing liposuction procedures. Four ASC culture conditions were investigated: ASCs cultured in basal Dulbecco's Modified Eagle Medium (DMEM); ASCs cultured in serum-free DMEM; ASCs cultured in serum-free DMEM/F12; and ASCs cultured in a CM from ARPE-19, a spontaneously arising cell line with a normal karyotype derived from a human RPE. Cell proliferation rate and viability were assessed by crystal violet and MTT assays at 1, 4

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Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines. No animals were used in the present study.

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and 8 d of culture. At the same time points, ASC neural differentiation was evaluated by immunocytochemistry and western blot analysis for typical neuronal and glial markers: Nestin, neuronal specific enolase (NSE), protein gene product (PGP) 9.5, and glial fibrillary acidic protein (GFAP).

RESULTS
Depending on the culture medium, ASC proliferation rate and viability showed some significant differences. Overall, less dense populations were observed in serum-free cultures, except for ASCs cultured in ARPE-19 serum-free CM. Moreover, a different cell morphology was seen in these cultures after 8 d of treatment, with more elongated cells, often showing cytoplasmic ramifications. Immunofluorescence results and western blot analysis were indicative of ASC neural differentiation. In fact, basal levels of neural markers detected under control conditions significantly increased when cells were cultured in ARPE-19 CM. Specifically, neural marker overexpression was more marked at 8 d. The most evident increase was observed for NSE and GFAP, a modest increase was observed for nestin, and less relevant changes were observed for PGP9.5.

CONCLUSION
The presence of growth factors produced by ARPE-19 cells in tissue culture induces ASCs to express neural differentiation markers typical of the neuronal and glial cells of the retina.

Key Words: Adipose-derived mesenchymal stem cells; Retinal pigment epithelium; Neural markers; Neural differentiation; Retina damage; Cell-based medicine

Core Tip: Neural-like differentiation of adipose-derived stem cells (ASCs) was tested using a conditioned medium from ARPE-19 cells, a cell line derived from human retinal pigment epithelium. Following this treatment, the expression of typical glial and neuronal markers increased in a time-dependent manner. Neural-like differentiated ASCs may represent a valuable tool for cell-based therapeutic approaches in the field of regenerative medicine for the treatment of eye diseases.

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INTRODUCTION

Mesenchymal stem cells (MSCs) have been widely investigated in the last two decades in order to develop cell-based therapeutic strategies for a variety of human pathologies including eye disease[1-4]. Based on their multipotent differentiation ability, MSCs of different sources (bone marrow, adipose tissue, umbilical cord) have been successfully differentiated not only into cells of mesodermal origin, but also into cells of different derivation, such as epithelial and neural cells[5,6]. In particular, adipose-derived stem cells (ASCs) have been increasingly explored because they offer numerous advantages: They can be obtained in a large amount from subcutaneous tissue with minimal discomfort for the donors; they feature a high proliferation rate; they can also be used for allogeneic transplantation because of their low immunogenicity.

In a recent study, we were able to induce pericyte-like differentiated human ASCs [7], suitable for the treatment of diabetic retinopathy, characterized by extensive pericyte loss. However, several other cell types may be affected in retinal diseases, such as Müller cells[8], astrocytes[9], and photoreceptors[4]. Moreover, the visual loss occurring in diabetic retinopathy or in glaucoma is related to retinal sensory dysfunction, mainly due to retinal ganglion cell (RGC) loss.

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The purpose of this study was to test whether growth of ASCs in serum free tissue culture medium conditioned by retinal pigment epithelium (RPE) could trigger their neural differentiation.

RPE is a specialized epithelium lying between the neural retina and the capillary lamina of the choroid[10]. Early in development, RPE is required for the normal growth of the eye. However, it is also fundamental to maintain the correct retina homeostasis also in adults[11].

It has multiple functions, such as absorption of light and protection against photo-oxidation, transport of nutrients, water, and ions. Other than playing a crucial role in the constitution of the outer blood-retina barrier, RPE cells govern differentiation and regeneration of photoreceptors and retinal progenitor cells through a variety of growth factors within the retinal stem cell niche[12]. RPE-secreted factors are able to rescue degenerating photoreceptors by prolonging their survival. In addition, they can transdifferentiate and give rise to photoreceptors, bipolar or multipolar (ganglionic and amacrine) cells[13]. In addition, RPE conditioned medium (CM) drives differentiation of retinal progenitor cells towards photoreceptors, depending on the cell density.

Indeed, the effects of human or porcine RPE cell CM on ASCs were tested in a work by Vossmerbaeumer *et al*[14], reporting a possible ASC differentiation toward the RPE lineage, as suggested by the increased expression of typical RPE markers such as bestrophin, cytokeratins 8 and 18, and RPE 65. However, a systematic study on neural marker expression was not carried out.

In the present work, ASC neural differentiation was induced by culture in CM from ARPE-19, a spontaneously arising cell line with a normal karyotype derived from human retinal pigmented epithelium[15]. In this way, ASCs would grow in an *in vitro* environment resembling the environment existing in the normal eye, without addition of chemical agents that might be potentially toxic. ASC differentiation was verified by immunocytochemical techniques and western blot analysis for nestin, neuronal specific enolase (NSE), protein gene product (PGP) 9.5 and glial fibrillary acidic protein (GFAP).

MATERIALS AND METHODS

ARPE-19 cultures and preparation of ARPE-19 CM

The human retinal pigment epithelial cell line (ARPE-19) was purchased from the American Type Culture Collection (CRL-2302™) and cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (ATCC 30-2006, Washington, DC, United States) containing 10% phosphate buffered saline (FBS) and 1% penicillin/streptomycin. For CM preparation, ARPE-19 cells were seeded and cultured until 80% confluence was reached, usually after 24 h, when the medium was replaced with fresh, serum-free, DMEM/F12. The day after, the medium was collected, filtered to remove debris and floating cells, and stored at −20 °C before further use.

ASC cultures

ASCs were isolated from adipose tissue, harvested from the subcutaneous region of four healthy female donors (32–38-years-old) undergoing liposuction procedures at the Cannizzaro Hospital, Catania (Italy). Lipoaspirate was obtained after donors signed a written informed consent to allow the use of the adipose tissue for experimental investigations, which were carried out in accordance with the Declaration of Helsinki. The protocol was approved by the local ethics committee (Comitato etico Catania1; Authorization n. 155/2018/PO).

Red blood cells and debris were removed by washing the raw lipoaspirate (50–100 mL) with sterile PBS (Invitrogen). It was then incubated for 3 h at 37 °C with DMEM containing 0.075% of type I collagenase (GIBCO 17100017, Thermo Fisher Scientific, Waltham, MA, United States). The collagenase was then inactivated by adding an equal volume of DMEM (Lonza 12-707F, Basel, CH) containing 10% FBS (DMEM/FBS) and the lipoaspirate was centrifuged for 10 min at 1200 rpm. After pellet resuspension in PBS, cells were filtered through a 100 μm nylon cell strainer (Falcon BD Biosciences, Milan, Italy). Following a final centrifugation/resuspension procedure, cells were plated in T75 culture flasks (Falcon BD Biosciences) with DMEM/FBS, 1% penicillin/streptomycin, 1% MSC growth supplement (MSCGS; ScienCell Research Laboratories, Milan, Italy). Cells were incubated at 37 °C with 5% CO_2 until confluence (about 80% of total flask surface) was reached. Cells were cultured for 3 passages before the subsequent procedures.

The MSC nature of ASCs used in the present study had been verified in previous studies, where cells of the same stock had been investigated[7]. Virtually the entire population (above 98% of cells) was immunopositive for typical MSC markers (CD44, CD73, CD90, and CD105), whereas only a few cells (less than 1%) were immunostained for typical hematopoietic stem cell markers (CD14, CD34, and CD45).

For the present investigation, four groups of ASC cultures were prepared. In the first group ASCs were maintained in DMEM/FBS (ASCs); in the second group, ASCs were cultured in serum-free DMEM (sfASCs); in the third group ASCs were cultured in serum-free DMEM/F12 (F12/ASCs); and in the fourth group, ASCs were grown in ARPE-19 CM (CM/ASCs). From each group, some samples were processed at 1 d of culture; other samples were processed at day 4; further samples were processed at day 8. At each time point, cell proliferation and viability assays, fluorescence immunocytochemistry and western blot procedures were carried out for specific signal detection.

Cell proliferation assay

The crystal violet assay was used to evaluate the proliferation rate of ASCs of each group at 1, 4 and 8 d of culture. To this purpose, cells were stained with 0.5% crystal violet solution in 20% methanol for 10 min. After photomicrographs were taken (Leica Microscope), the crystal violet was solubilized in 1% sodium dodecyl sulphate (SDS) and absorbance values were measured at 570 nm with a microplate reader (Synergy 2-BioTek). Each assay was carried out in triplicate, from three independent experiments.

Cell viability assay

Cell viability was evaluated in ASCs of each group at 1, 4 and 8 d of culture. To this purpose, 3-[4,5-dimethylthiazol-2-y l]-2,5-diphenyl tetrasodium bromide (MTT assay, Chemicon, Temecula, CA, United States) was added to each sample and incubated for 3 h at 37 °C. The supernatant was then removed and 100 μL Dimethyl Sulfoxide (DMSO) were used to dissolve the precipitate. Absorbance values were determined at 570 nm in a plate reader (Synergy 2-BioTek). Each assay was carried out in triplicate, from three independent experiments.

Immunofluorescence

Immunocytochemical staining was carried out following a protocol previously described[6]. Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde and incubated for 30 min with a 5% solution of normal goat serum (Sigma–Aldrich) in PBS containing 0.1% Triton (Sigma–Aldrich). They were then exposed overnight at 4 °C to primary antibodies: Mouse anti-nestin (1:100, Abcam, ab22035 Cambridge, MA, United States); mouse anti-NSE (1:100, Abcam ab16808); rabbit anti-PGP9.5 (1:100, Abcam ab108986), and mouse anti-GFAP (1:100; Novus Biologicals NB120-10062, Centennial, CO, United States). The following day, cells were washed with PBS and incubated for 60 min at room temperature with secondary antibodies conjugated to different fluorochromes: FITC conjugated goat anti-rabbit (Abcam ab96899) and/or Cy3-conjugated goat anti-mouse (Abcam ab96880). Finally, DAPI was applied for 10 min to stain cell nuclei. In each experiment, specificity of immunostaining was verified in some samples by omitting the primary antibody. Immunofluorescence was detected using a Leica DMRB Fluorescence Microscope. Digital images were acquired through a 40 × oil objective and a computer-assisted digital camera (Leica DFC 320).

Immunostaining quantification was carried out using the FIJI-ImageJ measure tool (NIH, Bethesda, MD, United States). At each time point, at least three samples of each group were examined. Three digital photomicrographs were randomly selected from each sample. Up to five immunofluorescent cells were analyzed from each photomicrograph. Values were calculated from the average grayscale intensity. For each cell, the integrated density, the cell area and the mean fluorescence value were assessed. Three replicate measurements were performed for each capture region. The same procedure was applied to three different background areas, close to the selected cell. The corrected total cell fluorescence (CTCF) was then calculated, using the following equation:

CTCF = Integrated density - (cell area × background mean fluorescence).

Percentages of immunopositive cells were estimated counting immunostained cells and DAPI-stained nuclei in randomly selected microscopic fields.

Western blot analysis

Immunoblots were carried out on samples of each treatment group (ASCs, sfASCs, F12/ASCs and CM/ASCs) at day 1, 4 and 8 of growth. Cells were trypsinized, centrifuged and resuspended in RIPA buffer (Life Technologies), in the presence of a

protease inhibitor cocktail (Sigma), serine/threonine phosphatase inhibitors (Sigma) and tyrosine protein phosphatase inhibitors (Sigma). Protein concentrations were determined by the BCA protein assay using BSA as the standard. Cell lysates (50 μg protein) were loaded onto 4%-20% SDS-PAGE, blotted and probed for different target proteins.

Membranes were incubated overnight at 4 °C with the same primary antibodies used for immunofluorescence: Mouse anti-nestin (1:1000); rabbit anti-PGP9.5 (1:2000); mouse anti-NSE (1:1500); and mouse anti-GFAP (1:1500). The following day, the membranes were incubated with the respective secondary antibodies (1:2000) for 1 h at room temperature, and the immunocomplexes were detected by the ChemiDoc™ Touch Imaging System (BIO-RAD). All blots were checked for equal loading by probing with GAPDH (rabbit, 1:1000; Cell Signaling). Densitometry analysis was performed using free software Image J (NIH, Bethesda, MD, United States).

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, United States). For each experimental condition, values are reported as mean ± SD. Differences between samples were assessed using two-way analysis of variance (two-way ANOVA) followed by post hoc Tukey's multiple comparisons test. *P* values of 0.05 or less were considered statistically significant. The statistical methods of this study were reviewed by Dr Vincenzo Guardabasso, Specialist in Public Health Statistics, University of Catania, Italy.

RESULTS

ASC morphology, proliferation, and viability

Depending on the culture medium, ASCs showed some significant differences. At day 1 (Figure 1A), all samples exhibited the typical fibroblast-like morphology. However, a decrease in population density was observed in serum-free cultures, especially in F12/ASCs. A lower decrease was seen in CM/ASCs. More marked differences were observed at day 8. At this time, a denser cell population was observed in control ASCs, still conserving the same shape as day 1. Moreover, a decreased population density was observed for ASCs cultured under serum-free conditions; however, this was less evident in CM/ASCs. It is worth noting that under the CM/ASC condition, a different cell morphology was apparent, with more elongated cells, often showing cytoplasmic ramifications. Data illustrated in representative pictures are supported by quantitative measurements, for cell proliferation (Figure 1B) and viability (Figure 1C).

ASC neural differentiation

Immunofluorescence results and western blot analyses indicated that a neural differentiation likely occurs when ASCs were cultured in ARPE-19 CM. Overall, although to a different extent, all neural markers increased their expression in a time-dependent fashion.

Nestin

Immunofluorescence photomicrographs (Figure 2) and western blot (Figure 3) analyses revealed that a basal level of nestin could be detected in a considerable portion (62%) of cells in all ASC samples at 1 d of culture (Table 1). At day 4, these basal levels were reduced in serum-free cultures (sfASCs and F12/ASCs), whereas comparable values were maintained in CM/ASCs. At day 8, basal nestin levels were still present in control ASCs, while they were strongly decreased in serum-free-cultures, especially in F12/ASCs. On the contrary, increased nestin levels were observed in CM/ASCs.

These observations were in agreement with quantitative immunofluorescence estimates (Figure 4). No evident changes were observed at day 1, except for a modest increase in CM/ASCs. At day 8, fluorescence intensity and percentages of immunopositive cells (Table 1) were lower in serum-free cultures, whereas both parameters were increased in CM/ASCs.

NSE

A similar trend was observed for NSE expression modifications (Figures 3 and 5). Comparable basal values were detected at day 1 in all ASC samples. At day 4 a decreased NSE expression was observed in sfASCs and F12/ASCs, whereas increased

Table 1 Percentage of immunostained cells of each sample for neural markers at day 1 and day 8 of culture

Marker	Day 1				Day 8			
	ASCs	sfASCs	F12/ASCs	CM/ASCs	ASCs	sfASCs	F12/ASCs	CM/ASCs
Nestin	62 ± 5	53 ± 4	51 ± 3	78 ± 4	74 ± 6	39 ± 2	31 ± 3	88 ± 4
NSE	63 ± 3	52 ± 6	54 ± 7	82 ± 3	61 ± 4	49 ± 3	50 ± 4	93 ± 5
PGP9.5	68 ± 4	65 ± 3	66 ± 5	75 ± 5	78 ± 5	62 ± 3	67 ± 6	89 ± 3
GFAP	55 ± 7	56 ± 5	53 ± 4	77 ± 3	53 ± 6	41 ± 5	45 ± 7	87 ± 5

ASCs: Control ASCs cultured in basal DMEM; CM/ASCs: ASCs cultured in serum-free DMEM/F12 conditioned from ARPE-19; F12/ASCs: ASCs cultured in serum-free DMEM/F12; sfASCs: ASCs cultured in serum-free DMEM. GFAP: Glial fibrillary acidic protein; NSE: Neuron specific enolase; PGP9.5: Protein gene product 9.5; ASCs: Adipose-derived stem cells; sfASCs: Serum-free adipose-derived stem cells; CM: Conditioned medium.

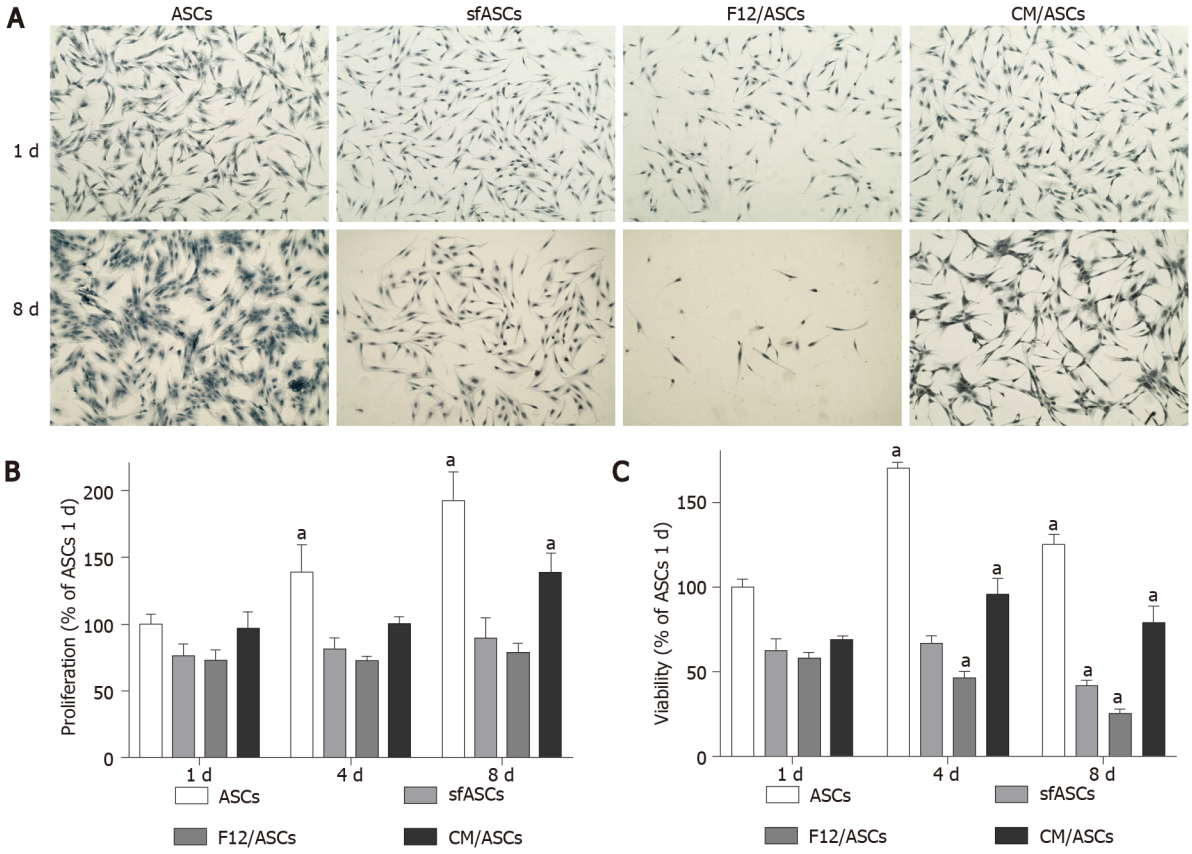

Figure 1 Crystal violet and MTT assays in different samples of adipose-derived stem cells cultures. A: Representative microphotographs of crystal violet staining of human adipose-derived stem cells (ASCs) at 1 d and 8 d of culture. ASCs: Control ASCs cultured in basal Dulbecco's Modified Eagle Medium (DMEM); sfASCs: ASCs cultured in serum-free DMEM; F12/ASCs: ASCs cultured in serum-free DMEM/F12; CM/ASCs: ASCs cultured in serum-free DMEM/F12 conditioned from ARPE-19. Scale bar: 100 μm; B and C: Proliferation rate (crystal violet assay, CV) (B) and cell viability (MTT assay) at 1, 4 and 8 d of culture (C). Absorbance values were determined at 570 nm. Values are expressed as mean ± SD of three independent experiments. Values are referred to the control ASC population, at each corresponding time point. $^{a}P < 0.05$ *vs* ASCs at day 1; Two-way ANOVA, followed by Tukey's multiple comparisons test. sfASCs: Serum-free adipose-derived stem cells; CM: Conditioned medium.

values were measured in CM/ASCs. A further increase was found at day 8 for CM/ASCs. Quantitative immunofluorescence measurements (Figure 4) and percentages of immunopositive cells (Table 1) confirmed that the most evident effects were detected for CM/ASCs, showing a marked increase at day 8.

PGP9.5

When compared to control ASCs, all detection methods showed that no evident differences were noted between the different samples, except for CM/ASCs at day 8 of treatment (Figures 3, 4 and 6 and Table 1)

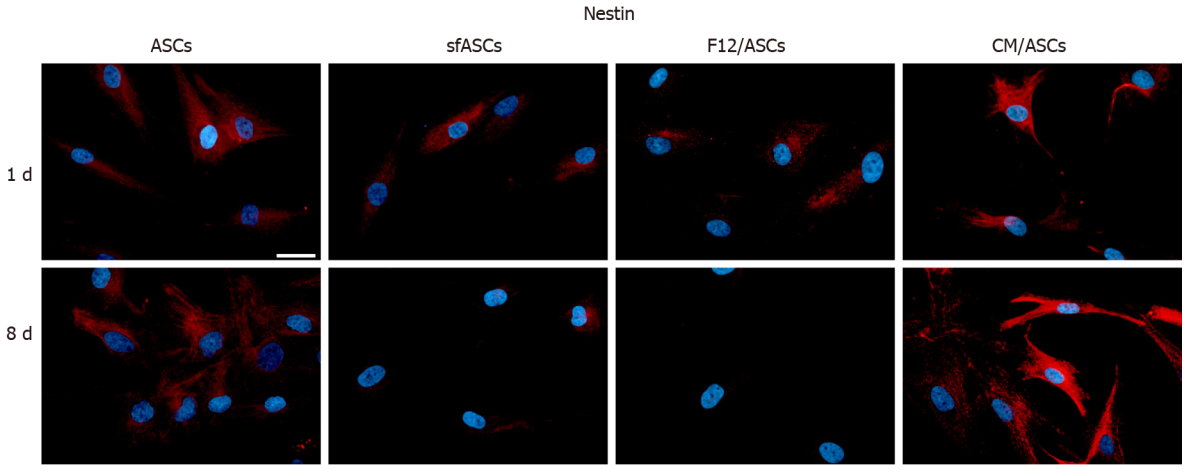

Figure 2 Nestin immunoreactivity in different samples of adipose-derived stem cells cultures. Nestin (red fluorescence) detected after 1 d and 8 d of culture of human adipose-derived stem cells (ASCs). ASCs: Control ASCs cultured in basal Dulbecco's Modified Eagle Medium (DMEM); sfASCs: ASCs cultured in serum-free DMEM; F12/ASCs: ASCs cultured in serum-free DMEM/F12; CM/ASCs: ASCs cultured in serum-free DMEM/F12 conditioned from ARPE-19. Blue fluorescence indicates DAPI staining of cell nuclei. Scale bar: 50 μm. sfASCs: Serum-free adipose-derived stem cells; CM: Conditioned medium.

GFAP

Basal GFAP values at day 1 and day 4 were similar in control and serum-free ASCs (Figures 3 and 7), whereas a significant increase was observed in CM/ASCs at both times. This increase was even more evident at day 8, whereas lower values were observed in serum-free cultures (sfASCs and F12/ASCs). These observations largely match the quantitative estimates reported in Figure 4 and Table 1.

DISCUSSION

As is well known, native ASCs exhibit a variety of cellular markers, some of them belonging to cell lineages quite different from each other. This characteristic is probably related to their multipotent differentiation ability, which is evident by the different cell types that can be obtained following different induction strategies. In fact, a wide range of differentiated cells can be obtained starting from native ASCs; from insulin-producing pancreatic cells[16] to neurons or glial cells[6].

The results presented here show that CM obtained from ARPE-19 can trigger differentiation of ASCs towards a neural-like phenotype. This is not unexpected, since RPE has tight interactions with the neural retina, secreting factors necessary for its homeostasis and function.

RPE derived growth factors include pigment epithelium-derived factor (PEDF), ciliary neurotrophic factor (CNTF)[17], basic fibroblast growth factor (FGF-2), epidermal growth factor (EGF), and nerve growth factor (NGF)[8,12,18]. Both FGF-2 and EGF have been shown to generate retinal neurons from human retinal precursor cells[19]. Secreted into the interphotoreceptor matrix, the neurotrophic factor PEDF induces antiapoptotic, antioxidative, and anti-inflammatory effects. The intraocular injection of PEDF delayed photoreceptor cell degeneration and apoptosis. Moreover, it also acted in neuronal differentiation and survival. PEDF-related effects may explain our observation about the different proliferation rates observed in the various samples examined in the present study. In fact, as already reported for human umbilical cord MSCs, the addition of PEDF significantly reduced apoptosis when cells were cultured in a serum-free medium[20]. In particular, the authors showed that this PEDF-induced apoptosis reduction was due to a decreased p53 expression. This is particularly important since this method allows for a significant cell expansion even in serum-free cultures, thus reducing safety related problems for possible clinical applications. ARPE-19 effects, weakly visible at day 1, was more pronounced at day 4 and, particularly, at day 8.

A panel of neural markers was chosen to verify the differentiating phenotype of ASCs under the described culture conditions. Nestin is an intermediate filament protein that is expressed in the early development stages of the central/peripheral nervous system, muscle and other tissues. During differentiation, it is downregulated and replaced by tissue-specific intermediate filament proteins[21]. PGP9.5 was

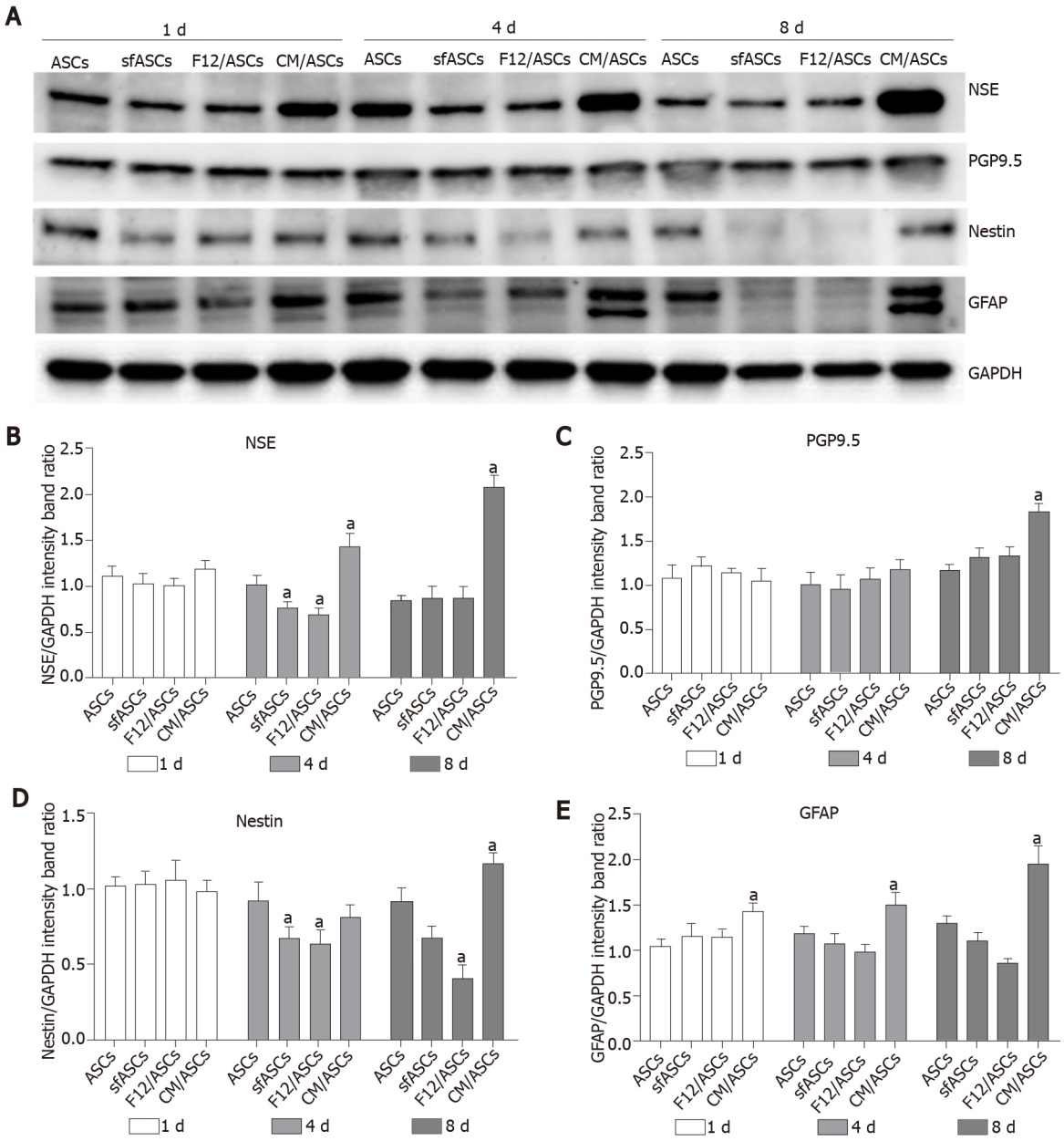

Figure 3 Western blot analysis of neural marker expression in different samples of adipose-derived stem cells cultures. A: Immunoblot analysis of whole-cell lysates at day 1, 4 and 8 of culture for NSE, PGP9.5, nestin, GFAP and GAPDH as internal control; B-E: ASCs: Control adipose-derived stem cells (ASCs) cultured in basal Dulbecco's Modified Eagle Medium (DMEM); sfASCs: ASCs cultured in serum-free DMEM; F12/ASCs: ASCs cultured in serum-free DMEM/F12; CM/ASCs: ASCs cultured in serum-free DMEM/F12 conditioned from ARPE-19. Quantitative data are illustrated in histograms. Values are expressed as mean ± SD obtained from three independent experiments. $^{a}P < 0.05$ *vs* ASCs of corresponding time point; Two-way ANOVA, followed by Tukey's multiple comparisons test. NSE: Neuron specific enolase; PGP9.5: Protein gene product 9.5; GFAP: Glial fibrillary acidic protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; sfASCs: Serum-free adipose-derived stem cells; CM: Conditioned medium.

originally detected as a "brain-specific protein", accounting for about 5% of total neuronal proteins[22,23]. NSE is currently considered a useful marker of neural maturation, being highly specific for neurons and peripheral neuroendocrine cells[24]. NSE may also induce neurotrophic functions as it controls neuronal survival, differentiation, and neurite regeneration[25,26]. GFAP expression is commonly considered specific of astrocytes[27], also present in activated Müller cells of the retina[28] and multipotent neural stem cells of the adult mammalian brain[29].

Several studies report the presence of these markers also within the mammalian retina, some of them at different stages of development and under different conditions. According to Mayer *et al*[30], nestin-positive cells in the normal retina represent a population of progenitor cells that differentiate to protect the structural integrity of the retina and RGCs. In the adult retina, they show morphological similarities to neural cells, such as RGCs, and Müller cells. Subpopulations of nestin-positive cells were also positive for GFAP. Nestin-positive cells are probably involved

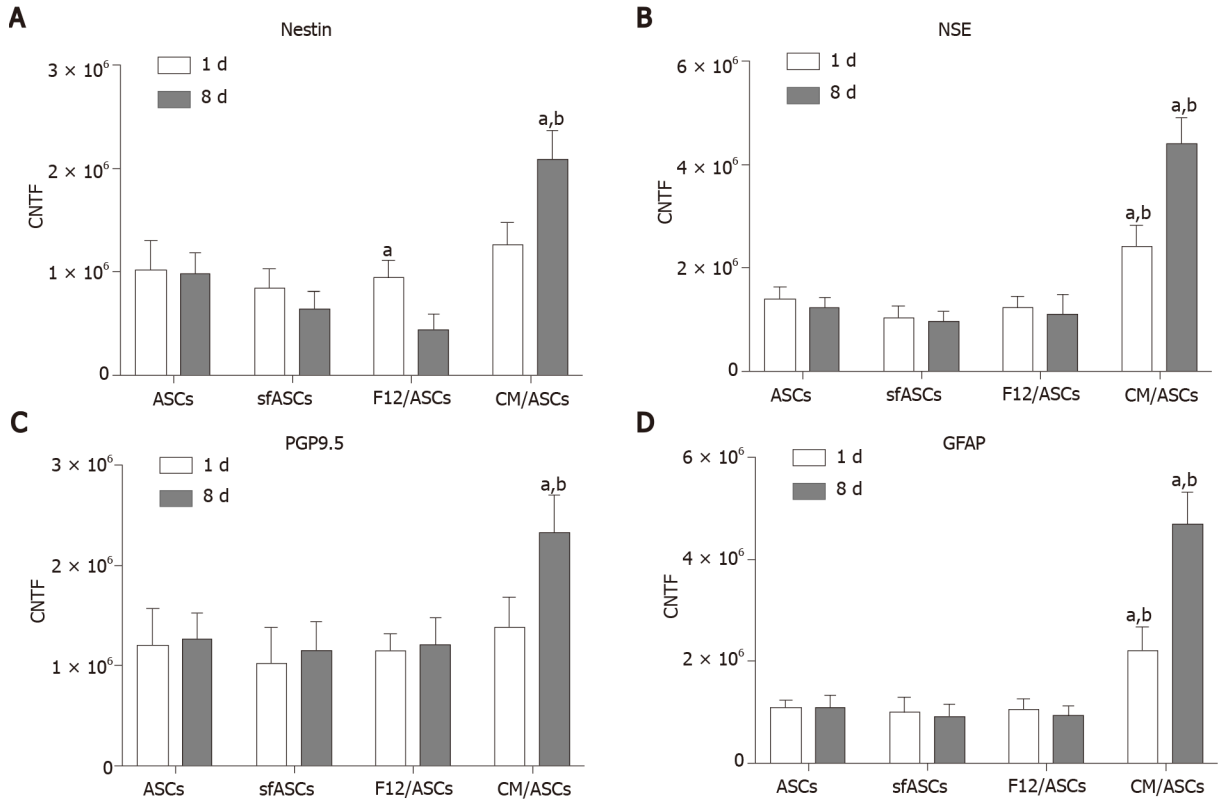

Figure 4 Immunofluorescence quantification for neural markers in each adipose-derived stem cells group at day 1 (white columns) and day 8 (dark columns). A: Nestin; B: NSE; C: PGP9.5; D: GFAP. ASCs: Control adipose-derived stem cells (ASCs) cultured in basal Dulbecco's Modified Eagle Medium (DMEM); sfASCs: ASCs cultured in serum-free DMEM; F12/ASCs: ASCs cultured in serum-free DMEM/F12; CM/ASCs: ASCs cultured in serum-free DMEM/F12 conditioned from ARPE-19. Bars represent CTCF mean values ± SD, obtained from at least three independent experiments. [a]Significant differences ($P < 0.01$) *vs* ASCs; [b]Significant differences ($P < 0.01$) between CM/ASCs and DMEM/F12. CTCF: Corrected total cell fluorescence; NSE: Neuron specific enolase; PGP9.5: Protein gene product 9.5; GFAP: Glial fibrillary acidic protein; sfASCs: Serum-free adipose-derived stem cells; CM: Conditioned medium.

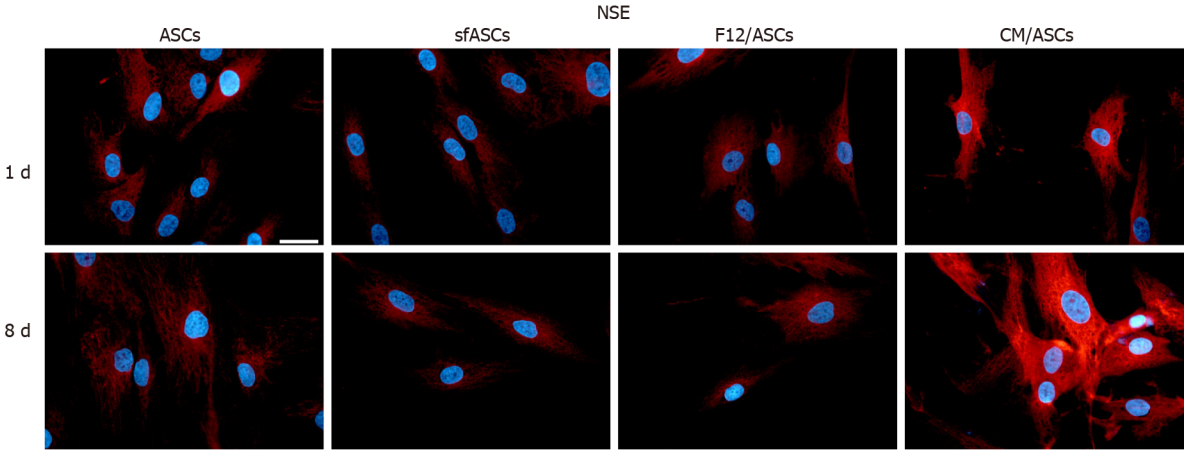

Figure 5 Neuronal specific enolase immunoreactivity in different samples of adipose-derived stem cells cultures. Neuronal specific enolase (red fluorescence) detected after 1 d and 8 d of culture of human adipose-derived stem cells (ASCs). ASCs: Control ASCs cultured in basal Dulbecco's Modified Eagle Medium (DMEM); sfASCs: ASCs cultured in serum-free DMEM; F12/ASCs: ASCs cultured in serum-free DMEM/F12; CM/ASCs: ASCs cultured in serum-free DMEM/F12 conditioned from ARPE-19. Blue fluorescence indicates DAPI staining of cell nuclei. Scale bar: 50 μm. sfASCs: Serum-free adipose-derived stem cells; CM: Conditioned medium; NSE: Neuronal specific enolase.

in regenerative processes, since their number increases following optic nerve transection[31]. PGP9.5 immunoreactivity has been detected in the retina of several mammalian species, especially in ganglion cells, suggesting that PGP9.5 can be used as a specific neuronal marker for these neurons[32]. In fact, PGP9.5 immunoreactivity was found in about 80% of ganglion cells retrogradely labeled after injection of peroxidase into the optic nerve[33]. Widely distributed in small to medium size

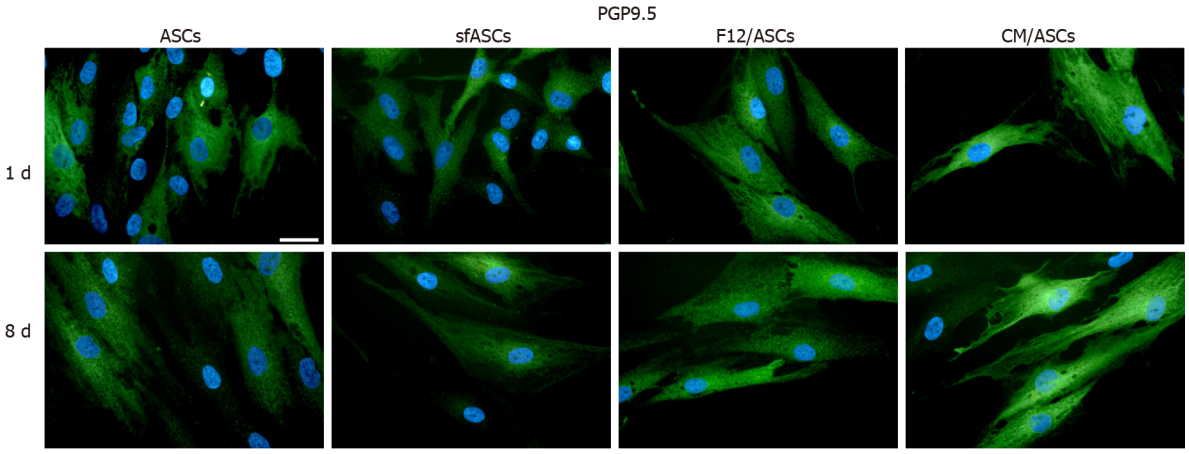

Figure 6 Protein gene product 9.5 immunoreactivity in different samples of adipose-derived stem cells cultures. Protein gene product 9.5 (green fluorescence) detected after 1 d and 8 d of culture of human adipose-derived stem cells (ASCs). ASCs: Control ASCs cultured in basal Dulbecco's Modified Eagle Medium (DMEM); sfASCs: ASCs cultured in serum-free DMEM; F12/ASCs: ASCs cultured in serum-free DMEM/F12; CM/ASCs: ASCs cultured in serum-free DMEM/F12 conditioned from ARPE-19. Blue fluorescence indicates DAPI staining of cell nuclei. Scale bar: 50 μm. sfASCs: Serum-free adipose-derived stem cells; CM: Conditioned medium; PGP: Protein gene product.

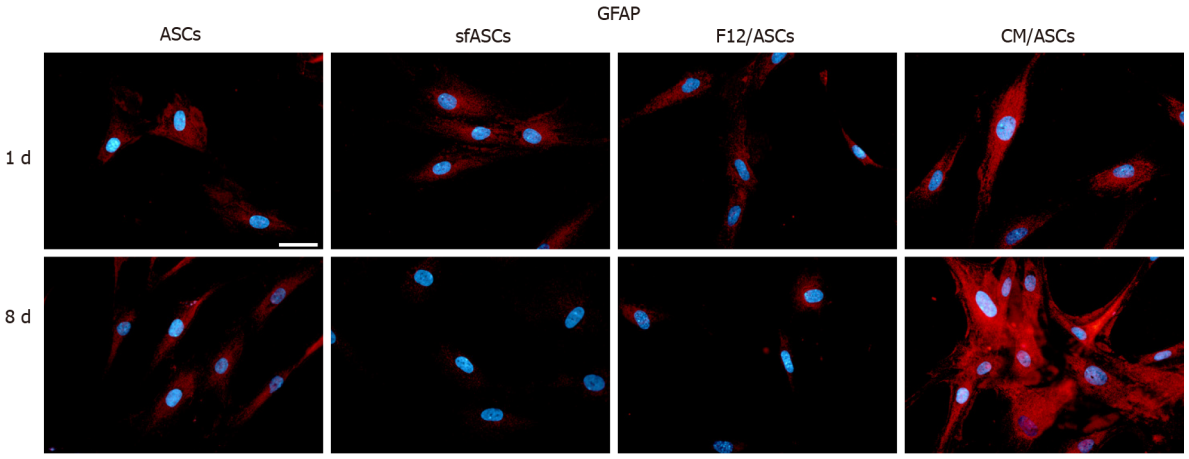

Figure 7 Glial fibrillary acid protein immunoreactivity in different samples of adipose-derived stem cells cultures. Glial fibrillary acid protein (red fluorescence) detected after 1 d and 8 d of culture of human adipose-derived stem cells (ASCs). ASCs: Control ASCs cultured in basal Dulbecco's Modified Eagle Medium (DMEM); sfASCs: ASCs cultured in serum-free DMEM; F12/ASCs: ASCs cultured in serum-free DMEM/F12; CM/ASCs: ASCs cultured in serum-free DMEM/F12 conditioned from ARPE-19. Blue fluorescence indicates DAPI staining of cell nuclei. Scale bar: 50 μm. sfASCs: Serum-free adipose-derived stem cells; CM: Conditioned medium; GFAP: Glial fibrillary acid protein.

ganglion cells, it is suggested that PGP9.5 modulates the early stages of retina development[34]. Experiments in rats show that NSE immunopositive neurons can be clearly detected in the retina only during embryonic development and early neonatal stages[35]. The first appearance of NSE immunoreactivity was identified in pigment epithelium, then in ganglion cells, photoreceptors and amacrine cells. Further retinal neurons became NSE immunopositive by postnatal day 14. It is suggested that NSE expression occurs in retinal neurons just after their migration to their final location and before establishing synaptic contacts. High GFAP levels in the mammalian retina during the first neonatal week rapidly decline during animal growth. In fact, in the adult organism, only astrocytes are GFAP-positive, while Müller cells only weakly express GFAP. However, high levels of GFAP can be detected in Müller cells following photoreceptor degeneration or in cases of retinal degeneration/detachment. It is possible that GFAP upregulation occurs in activated "dedifferentiating" Müller cells because of a disruption of normal neuron-glia interactions[28].

Overall, it can be speculated that, even though some of these markers may be found in tissues different from the nervous system, their increased expression in morphologically changed cells induced by ARPE-19 CM is suggestive of ASC neural differentiation. Vossmerbaeumer *et al*[14] reported less induction of GFAP and nestin levels in ASCs exposed to pig-derived primary RPE-CM, in a study mainly designed to monitor

RPE markers, while neural markers were only marginally explored, to exclude neural stem cell contamination in their ASC samples. Possible differences could be related to a different antibody sensitivity and/or different experimental procedures. In fact, the lack of nestin immunostaining in their samples was contradicted by their quantitative real-time polymerase chain reaction results that, also in primary ASC cultures, revealed a basal nestin level. Moreover, this basal expression was found "unexpectedly" increased after porcine RPE CM treatment. In the present study, a systematic investigation by immunostaining and western blot analyses showed that an increased expression of both GFAP and nestin was consistently observed in a time dependent manner. In fact, although some differences could already be noted at day 1, they were more clearly appreciable at day 8. It is important to point out that striking differences were observed between basal F12/ASCs and CM/ASCS. In fact, since the same culture medium was used in both cases, the differences observed must be attributed to the release of soluble factors or extracellular vesicles by ARPE-19 during their growth. In this respect, it should be pointed out that serum-free cultures were also preferred to avoid interferences on ASC differentiation properties between factors present in ARPE-19 CM and FBS[36].

Since both neuronal and glial markers were found overexpressed in the same cell population, a likely possibility is that neural-like differentiating ASCs might still be at early stages of differentiation, similarly to neural progenitor cells, where both types of markers normally coexist[37-39]. An alternative explanation is that this might be a combined effect of the factors present in the CM and the particular *in vitro* situation, in the absence of a dynamic physiological environment, which would more specifically address the cell differentiation fate. It is reasonable to hypothesize that under *in vivo* conditions, on the basis of real microenvironment cues, their fate would be more precisely traced. For the same reason, some neural markers such as GFAP and NSE might be more expressed in neural-like differentiating ASCs. In fact, high levels of these markers, other than in response to retina damage, can be physiologically found at early stages of development.

Since different neural elements are present within a functional retina, further investigation will be carried out by using more specific markers to better clarify the type of neural cells into which ASCs preferentially differentiate. Moreover, it will be interesting to identify which component (growth factors and soluble molecules) might be responsible for the effects described in the present work. Finally, the presence of extracellular vesicles in ARPE-19 CM cannot be excluded and will be investigated in future studies.

CONCLUSION

ASC neural-like differentiation obtained by the protocol used in the present study offers some advantages. ASCs can be easily isolated for both autologous and heterologous use. A CM from an RPE cell line may closely mimic the physiologic environment of a functional retina. The use of a serum-free medium helps to meet the requests of regulatory authorities for the development of safe clinical applications.

ARTICLE HIGHLIGHTS

Research background

Based on their multipotent differentiation ability, mesenchymal stem cells (MSCs) have been widely investigated in the last two decades in order to develop cell-based therapeutic strategies for a variety of human pathologies including eye disease.

Research motivation

In many cases, available therapeutic approaches are not satisfactory to counteract the loss of retinal cells. Thus, administration of pre-differentiated MSCs may produce beneficial outcomes and improve the quality of life of patients suffering ocular diseases.

Research objectives

The aim of the investigation was to obtain a neural-like differentiation of adipose-derived stem cells (ASCs) using a serum-free culture medium, resembling the physiologic eye microenvironment.

Research methods

A serum-free conditioned medium (CM) from ARPE-19, a cell line derived from human retinal pigment epithelium, has been used to promote ASC neural differentiation. Immunofluorescence and western blot analysis were used to evaluate modifications of typical neural marker expression: Nestin, neuronal specific enolase, protein gene product 9.5, and glial fibrillary acidic protein.

Research results

Neural marker expression was increased in a time-dependent manner. In fact, CM effects were particularly evident after 8 d of treatment. Moreover, cell proliferation and viability were favored by the presence of ARPE-19 CM.

Research conclusions

The method adopted in the present study provided encouraging results to develop cell-based strategies for ocular diseases characterized by neural cell loss or degeneration.

Research perspectives

At the next stage of the study, neural-like pre-differentiated ASCs would be implanted in rodent models of ocular diseases to verify their survival rate and possible beneficial effects.

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ORIGINAL ARTICLE

Basic Study

MKK7-mediated phosphorylation of JNKs regulates the proliferation and apoptosis of human spermatogonial stem cells

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Author contributions: Zhu WB designed the study and supervised the laboratory experiments; Huang ZH conducted the experiments and drafted the manuscript; Huang C, Ji XR, Zhou WJ and Luo XF assisted with the experiments and sample collection; Liu Q, Tang YL and Gong F performed the analysis with constructive discussion and contributed analysis tools.

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Abstract

BACKGROUND

Human spermatogonial stem cells (SSCs) are the basis of spermatogenesis. However, little is known about the developmental regulatory mechanisms of SSC due to sample origin and species differences.

AIM

To investigates the mechanisms involved in the proliferation of human SSC.

METHODS

The expression of mitogen-activated protein kinase kinase 7 (MKK7) in human testis was identified using immunohistochemistry and western blotting (WB). MKK7 was knocked down using small interfering RNA, and cell proliferation and apoptosis were detected by WB, EdU, cell counting kit-8 and fluorescence-activated cell sorting. After bioinformatic analysis, the interaction of MKK7 with c-Jun N-terminal kinases (JNKs) was verified by protein co-immunoprecipitation and WB. The phosphorylation of JNKs was inhibited by SP600125, and the phenotypic changes were detected by WB, cell counting kit-8 and fluorescence-activated cell sorting.

RESULTS

MKK7 is mainly expressed in human SSCs, and MKK7 knockdown inhibits SSC proliferation and promotes their apoptosis. MKK7 mediated the phosphorylation

the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

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of JNKs, and after inhibiting the phosphorylation of JNKs, the phenotypic changes of the cells were similar to those after MKK7 downregulation. The expression of MKK7 was significantly downregulated in patients with abnormal spermatogenesis, suggesting that abnormal MKK7 may be associated with spermatogenesis impairment.

CONCLUSION
MKK7 regulates the proliferation and apoptosis of human SSC by mediating the phosphorylation of JNKs.

Key Words: MKK7; Spermatogonial stem cell; Proliferation; Apoptosis; JNKs

Core Tip: This study demonstrated that mitogen-activated protein kinase kinase 7 regulates the proliferation and apoptosis of human spermatogonial stem cells by mediating the phosphorylation of c-Jun N-terminal kinases *in vitro*. In addition, we found that mitogen-activated protein kinase kinase 7 was significantly downregulated in patients with reproductive disorders, suggesting that mitogen-activated protein kinase kinase 7 may be associated with human spermatogenesis.

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INTRODUCTION

Infertility is an important global health problem that affects approximately 15% of couples of childbearing age[1]. For about 50% of such couples, male factors are a contributing cause[2]. There are many possible types of male subfertility, including idiopathic, acquired and congenital[3]. Although most male infertility problems can be solved through assisted reproductive technology, there are still some patients who are unable to have genetic offspring, especially those with non-obstructive azoospermia (NOA)[4]. Thus, it is necessary to find other effective treatments, such as spermatogonial stem cell (SSC) transplantation and *in vitro* spermatogenesis.

SSCs are present on the basement membrane of the seminiferous tubules of the testis, and these cells self-renew and differentiate for life-long spermatogenesis[5]. Intricate molecular and cellular interactions form the niche for SSC development[6]. SSC self-renewal is promoted by the secretion of glial cell line-derived neurotrophic factor (GDNF) from the niche[7]. *In vitro*, GDNF and fibroblast growth factor 2 (FGF2) were observed to function synergistically to promote the growth of undifferentiated spermatogonia[8]. However, the function of basic FGF in SSC maintenance *in vivo* remains poorly defined[9]. Common molecules are activated by GDNF and FGF2 through Src family kinases, resulting in Ras activation[10]. Glucocorticoid-induced leucine zipper protein antagonizes Ras and stimulates the protein kinase B and MAPK/ERK kinase pathways[11]. When active protein kinase B or mitogen-activated protein kinase kinase 1 (a downstream molecule of the MAPK/ERK kinase pathway) were overexpressed, SSCs transfected with protein kinase B or mitogen-activated protein kinase kinase 1 could proliferate only in the presence of FGF2 or GDNF[12,13], respectively. Maintenance of the self-renewing state requires these pathways to upregulate certain genes, including ETS variant transcription factor 5, LIM homeobox 1, BCL6B transcription repressor and Nanos C2HC-type zinc finger 2[14].

Although studies have revealed a number of regulators of SSC self-renewal in mice, the regulatory mechanisms of SSCs are significantly different in humans and rodents [15]; these results cannot be repeated in human SSCs. In mouse, stemness occurs within the single spermatogonia; by contrast, the chains and pairs of spermatogonia are believed to represent committed progenitors[16,17]. In humans, Adark sperma-

togonia are regarded as the actual stem cells and slowly produce Apale spermatogonia, which have enough self-renewal capacity to maintain the stem cell pool[18-20]. Moreover, in mouse, the seminiferous epithelium cycle usually comprises 12 stages; however, only 6 stages have been distinguished in humans[21,22]. Notably, mouse and human SSCs have some common biomarkers; however, others differ considerably. For example, octamer-binding protein 4, a marker of mouse SSCs, is absent in humans[23,24]. Likewise, GDNF family receptor alpha 1 (GFRA1) positive spermatogonia are rare in mouse seminiferous tubules but abundant in humans[15].

Mitogen-activated protein kinase kinase 7 (MKK7) is an activator of c-Jun N-terminal kinase (JNK). MKK7 directly activates JNK1 and JNK2 (stressactivated protein kinases) *via* phosphorylation[25-27]. JNK signaling has an important function in self-renewal, apoptosis and cell proliferation and was reported to be associated with reactive oxygen species-mediated mouse SSC self-renewal[28]. In the present study, we found that MKK7 was mainly expressed in human SSCs [GFRA1$^+$/proliferating cell nuclear antigen (PCNA)$^+$/KIT proto-oncogene, receptor tyrosine kinase (KIT)$^-$] with proliferative activity. Deletion of MKK7 promoted human SSC apoptosis and impaired their proliferation. We further confirmed that MKK7 regulates SSC proliferation by inducing JNK1/JNK2/JNK3 phosphorylation. Additionally, MKK7 levels were confirmed to be decreased in patients with NOA compared with those in patients with obstructive azoospermia with normal spermatogenesis, particularly in patients with spermatogonial or spermatocyte maturation arrest. Our results reveal the molecular mechanisms responsible for human SSC proliferation and apoptosis and provide the basis for further research into the etiology, molecular diagnosis and therapy for male infertility.

MATERIALS AND METHODS

Collection of human testes samples

The Ethics Committee of the Reproductive & Genetic Hospital of CITICXiangya, Basic Medical Science School, Central South University approved and supervised the present study (approval No.: LL-SC-2020-028). The samples used in the experiments were provided by donors who supplied informed consent. All testis samples were derived from patients undergoing microdissection testicular sperm extraction, and patients with spermatogenic failure because of known hereditary factors, such as Klinefelter syndrome and Y chromosome microdeletions, were excluded. We collected a total of 16 testicular biopsies weighing 30-50 mg and classified them according to the results of hematoxylin-eosin staining, including normal, spermatogenic failure and Sertoli cell only syndrome. Testis tissue samples were rinsed thrice in Dulbecco's modified Eagle's medium (DMEM) comprising 10 mL/L streptomycin and penicillin and then fixed with 40 g/L paraformaldehyde or stored in liquid nitrogen.

Culture of a human SSC line

The method described by Hou *et al*[29] was used to establish a human SSC line, which was a gift from He ZP, Hunan Normal University, Changsha. Human SSCs were cultured in DMEM/F12 (Gibco, Grand Island, NY, United States) containing 100 mL/L fetal bovine serum (Gibco) and 100 unit/mL streptomycin and penicillin (Invitrogen, Carlsbad, CA, United States) at 34 °C in a 50 mL/L CO_2 incubator. Every 4 d, the cells were passaged using 0.53 mmol/L EDTA with 0.5 g/L trypsin (Invitrogen).

Extraction of RNA, reverse transcription polymerase chain reaction and quantitative real-time polymerase chain reaction

RNAiso Plus reagent (Takara, Kusatsu, Japan) was used to extract total RNA from cells following the supplier's protocol. Total RNA quality and concentration were measured using a Nanodrop instrument (Thermo Scientific, Waltham, MA, United States). The total RNA was converted to cDNA *via* reverse transcription PCR using a First Strand cDNA Synthesis Kit (Thermo Scientific) and following a previously described method [30]. The cDNA was then subjected to quantitative real-time PCR (qPCR) using SYBR Premix Ex Taq II (Takara) and comprising the following reaction conditions: 95 °C for 5 min; followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 52-60 °C for 45 s (see Supplementary Table 1 for the specific annealing temperatures) and elongation at 72 °C for 45 s. The negative control comprised RNA without reverse transcription but with qPCR. An Applied Biosystems ABI Prism 7700 system (Applied Biosystems, Foster City, CA, United States) was used to perform qPCR on triplicate samples.

The amplicons were separated *via* electrophoresis through 20 g/L agarose gels with ethidium bromide visualization. On the recorded images, chemiluminescence was used to analyze the band intensities (Chemi-Doc XRS, Bio-Rad, Hercules, CA, United States). The data were normalized to the expression of beta actin using the comparative cycle threshold method to calculate the relative mRNA expression level for each sample[31]. The designed primer sets for qPCR are listed in Supplementary Table 1.

Immunocytochemistry, immunohistochemistry and immunofluorescence

The human SSCs were rinsed thrice using cold phosphate buffer saline (PBS) (Gibco) and subjected to fixation for 15 min in 40 g/L paraformaldehyde. After a further three rinses with PBS, the cells were permeabilized for 10 min using 2.5 mL/L Triton X-100 (Sigma, St. Louis, MO, United States). The cells were blocked for 1 h at room temperature in 5% bovine serum albumin before being incubated with primary antibodies overnight at 4 °C. Supplementary Table 2 provides the detailed information regarding the antibodies used. After extensive washes with PBS, Alexa Fluor 488-conjugated immunoglobulin (Ig) G or Alexa Fluor 594-conjugated IgG were used as the secondary antibodies, and the nuclei were stained using 4,6-diamidino-2-phenylindole. Images were captured using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Xylene was used to deparaffinize testis sections, which were then rehydrated using a graded series of ethanol concentrations. Antigens were then retrieved by heating the samples at 98 °C for 18 min in 0.01 mol/L sodium citrate buffer. For immunohistochemistry but not for immunofluorescence, endogenous peroxidase activity was blocked using 30 mL/L H_2O_2 (Zsbio, Beijing, China). The sections were then subjected to permeabilization for 15 min using 2.5 mL/L Triton X-100 (Sigma). The sections were blocked at room temperature for 1 h using 50 mL/L bovine serum albumin for 1 h and then incubated with primary antibodies overnight at 4 °C. Following extensive washing using PBS for immunohistochemistry, the sections were incubated at room temperature for 1 h with horseradish peroxidaseconjugated secondary antibodies, and then chromogen detection was carried out using a 3,3′-diaminobenzidine chromogen kit (Dako, Glostrup, Denmark). The sections were finally stained with hematoxylin. For immunofluorescence, the sections were incubated at room temperature for 1 h with Alexa Fluorconjugated secondary antibody, and the nuclei were stained with 4,6-diamidino-2-phenylindole. The images were captured under a Zeiss microscope.

Western blotting and immunoprecipitation

Testis samples and human SSCs were ground and lysed on ice for 30 min using radioimmunoprecipitation assay lysis buffer (Thermo Scientific) and then subjected to centrifugation at 12000 *g* to produce clear lysates. A BCA kit (Thermo Scientific) was then used to determine the protein concentration in the lysates. Primary antibodies or control rabbit IgG were added to cell lysates and incubated at 4 °C overnight. The next day the supernatants were added with Protein G magnetic beads and incubated at 4 °C for 2 h. The samples were washed three times with washing buffer, the beads were pelleted magnetically, and then resuspended and boiled at 95 °C for 5 min. For each sample, 30 micrograms of total protein extracts were subjected to SDS-PAGE (Bio-Rad), and western blotting was performed following a previously published protocol [30]. Supplementary Table 2 details the antibodies used and their dilution ratios. Chemiluminescence (Bio-Rad) was used to visualize the intensities of the immunoreactive protein bands.

Small interfering RNA transfection

RiboBio (Guangzhou, China) synthesized the small interfering RNA (siRNA) sequences targeting human *MKK7* mRNA, which are listed in Supplementary Table 3. Scrambled siRNAs were used as negative controls. Lipofectamine 3000 (Life Technologies, Carlsbad, CA, United States) was used to transfect the control-siRNA or *MKK7*-siRNAs (both 100 nmol/L) into human SSCs, following the manufacturer's instructions. At 48 h after transfection, the cells were harvested to evaluate the changes in the expression levels of genes and proteins.

Cell counting kit-8 assay

After siRNA transfection, human SSC proliferation was detected using a cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) following the manufacturer's protocol. Briefly, 100 mL/L CCK-8 reagent replaced the cell culture medium, and the cells were incubated for 3 h. A microplate reader (Thermo Scientific) was then used to

measure the absorbance at 450 nm.

EdU incorporation assay

Human SSCs were seeded at 5000 cells/well in a 96-well plate; each well contained DMEM/F12 medium containing 50 μmol/L EdU (RiboBio). The cells were cultured for 12 h, washed using DMEM, and subjected to fixation using 40 g/L paraformaldehyde. Cell neutralization was achieved using 2 mg/mL glycine, followed by permeabilization for 10 min using 5 mL/L Triton X-100 at room temperature. Apollo staining reaction buffer was then used to reveal the EdU staining. Cell nuclei were stained using Hoechst 33342. A fluorescence microscope (Zeiss) was used to capture images, and the positive rate of EdU staining was calculated by counting at least 500 cells.

Staining with Annexin V/propidium iodide and flow cytometry

Human SSCs were transfected with siRNAs for 48 h and then assessed for their apoptotic percentage. Cells were digested with trypsin, washed twice using cold PBS, and collected by centrifugation. Cells (at least 10^6) were resuspended in Annexin V Binding Buffer (BD Biosciences, Franklin Lakes, NJ, United States) following the manufacturer's instructions. Then, 5 μL of Annexin Vconjugated Allophycocyanin and 10 μL of propidium iodide solution were added to the cells and incubated at room temperature for 15 min in the dark. The cells were then subjected to C6 flow cytometry analysis (BD Biosciences).

Terminal deoxynulceotidyl transferase nick-end-labeling assay

An In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) was used to determine the percentage of apoptosis among human SSCs, following the manufacturer's instructions. Proteinase K (20 mg/mL) was used to treat the cells for 15 min at room temperature. Thereafter, the cells were incubated with fluorescein isothiocyanate-12-deoxyuridine 5'-triphosphate labeling/terminal deoxynucleotidyl transferase enzyme buffer in the dark for 1 h. Cell nuclei were then labeled using 4,6-diamidino-2-phenylindole. The negative control cells were treated with PBS without the terminal deoxynucleotidyl transferase enzyme. At least 500 cells were evaluated per sample under a Zeiss fluorescence microscope.

Statistical analysis

All the statistical review of this study was performed by a biomedical statistician. GraphPad Prism 8.0 (GraphPad software, La Jolla, CA, United States) was used for the descriptive and statistical analyses. All experiments were performed at least in triplicate, and all data are presented as the mean ± SD. A *t*-test was used to calculate the statistical difference between two groups, with $P < 0.05$ being considered as statistically significant.

RESULTS

In humans, MKK7 is mainly expressed in SSCs

To explore MKK7 function, we first examined its expression in the adult human testis. The MKK7 protein level was measured in testis samples from patients with obstructive azoospermia with normal spermatogenesis using western blotting (Figure 1A). We further analyzed the localization of MKK7 in testicular tissue. MKK7 was mainly found in the cytoplasm of cells close to the seminiferous tubule basement membrane (Figure 1B), indicating that it might be expressed in SSCs. The results of double immunofluorescence staining showed that most MKK7-expressing cells were DEAD-box helicase 4-positive germ cells, and about 76% of MKK7 positive germ cells expressed GFRA1. Interestingly, about 90% of MKK7-expressing cells were PCNA positive (Figure 1C). Taken together, the results suggested that MKK7 was mainly expressed in SSCs and might be involved in cell proliferation.

MKK7 knockdown inhibits human SSC proliferation

We then used a human SSC cell line to explore the role of MKK7 in SSC proliferation. First, quantitative real time PCR was used to confirm the identity of the human SSC line. The results indicated that the cells expressed many markers of human SSCs, including *GFRA1*, Thy-1 cell surface antigen, ubiquitin C-terminal hydrolase L1 and DEAD-box helicase 4, while a hallmark of human Sertoli cells (encoding SRY-box transcription factor 9) was not detected (Supplementary Figure 1A). The results of

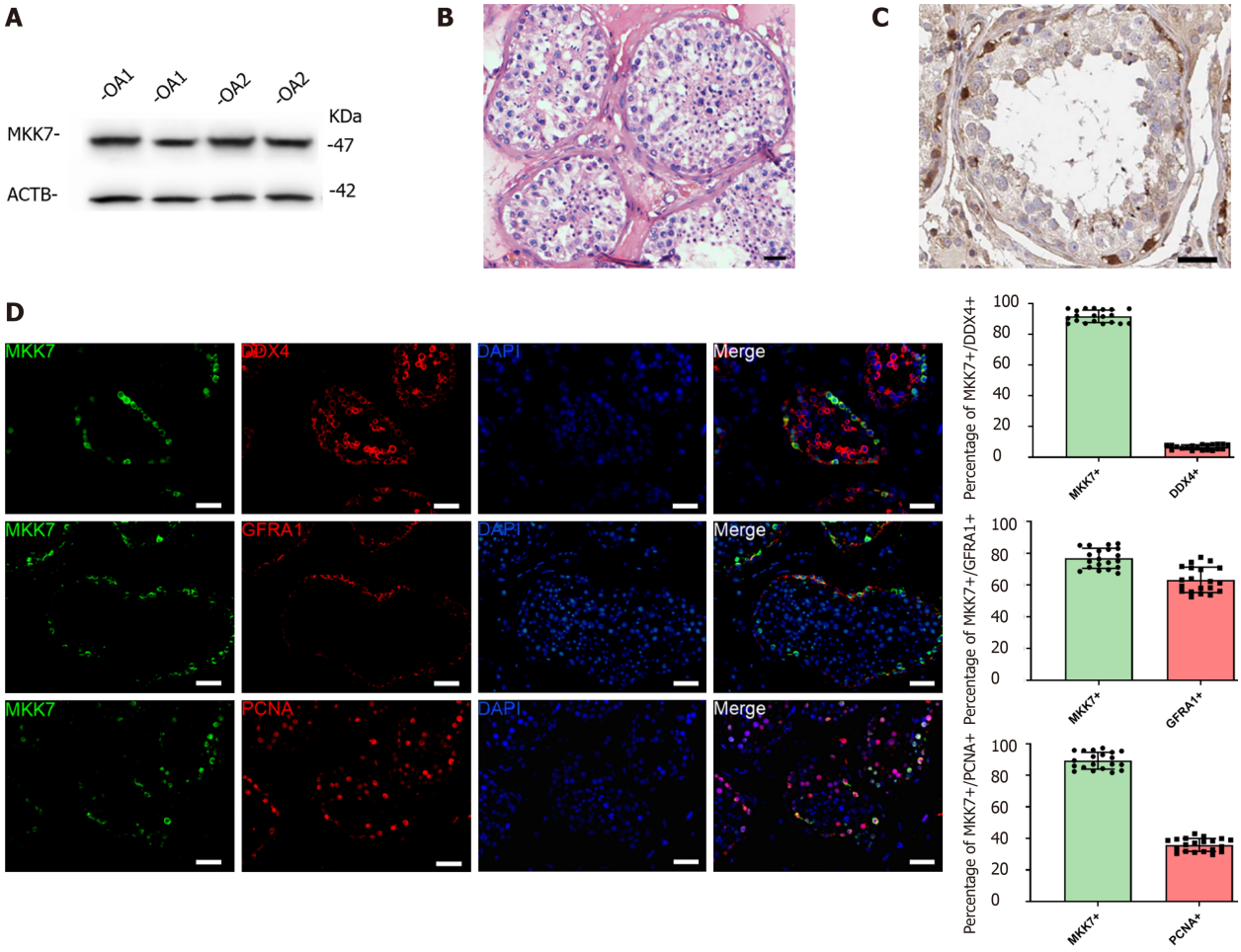

Figure 1 Mitogen-activated protein kinase kinase 7 was mainly expressed in human spermatogonial stem cells. A: Mitogen-activated protein kinase kinase 7 (MKK7) was detected in samples from 2 patients with obstructive azoospermia (OA) using western blotting; B: Hematoxylin-eosin staining of testis with normal spermatogenesis; C: Immunohistochemistry revealing the cellular localization of MKK7 in normal testis; D: Double immunostaining showing the co-expression of MKK7 with DEAD-box helicase 4 (DDX4), glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRA1) and proliferating cell nuclear antigen (PCNA) in normal human testis and the proportion of MKK7+ cells with DDX4, GFRA1 and PCNA expression; at least 20 seminiferous tubules were counted. Scale bars in B, C, and D: 50 μm. ACTB: Beta actin.

immunofluorescence also showed that almost all cells expressed SSC markers, such as GFRA1, ubiquitin C-terminal hydrolase L1 and Thy-1 cell surface antigen (Supplementary Figure 1B). These results suggested that the SSC line had similar properties to primary SSCs.

To explore the function of MKK7 in SSCs, siRNA-triggered knockdown of *MKK7* was performed in the human SSC line. We examined the knockdown efficiency using quantitative real time PCR (Figure 2A) and western blotting (Figure 2B and C), which showed that MKK7 expression was attenuated by *MKK7*-siRNA1, *MKK7*-siRNA2 and *MKK7*-siRNA3. *MKK7*-siRNA3 showed the best knockdown effect. We then investigated the proliferative ability of *MKK7*siRNA3-transfected SSCs using the CCK-8 assay (Figure 2D). We observed that *MKK7* knockdown repressed SSC proliferation at 2 d to 5 d after transduction. We also found that the level of PCNA (a cell proliferation hallmark) was significantly reduced (Figure 2E and F). Likewise, at 48 h after transfection, we examined cellular DNA synthesis using an EdU assay. The proportion of EdU-positive cells was decreased compared with that in the control siRNA transfected cells (35.73% ± 0.64% *vs* 22.05% ± 1.58%, $P < 0.05$) (Figure 2G and H).

MKK7 deficiency promoted human SSC apoptosis

Next, Annexin V/propidium iodide staining and flow cytometry were used to further examine cell apoptosis. *MKK7* silencing increased both early and late apoptosis of the human SSC line compared to that in the cells transfected with the controlsiRNA (early apoptosis: 5.17% ± 0.37% *vs* 0.62% ± 0.19%, $P < 0.05$; late apoptosis: 4.58% ± 0.40% *vs* 0.84% ± 0.09%, $P < 0.05$) (Figure 3A-C). Similarly, the results of terminal deoxynucleotidyl transferase nick-end-labeling (TUNEL) assay displayed that the proportion of

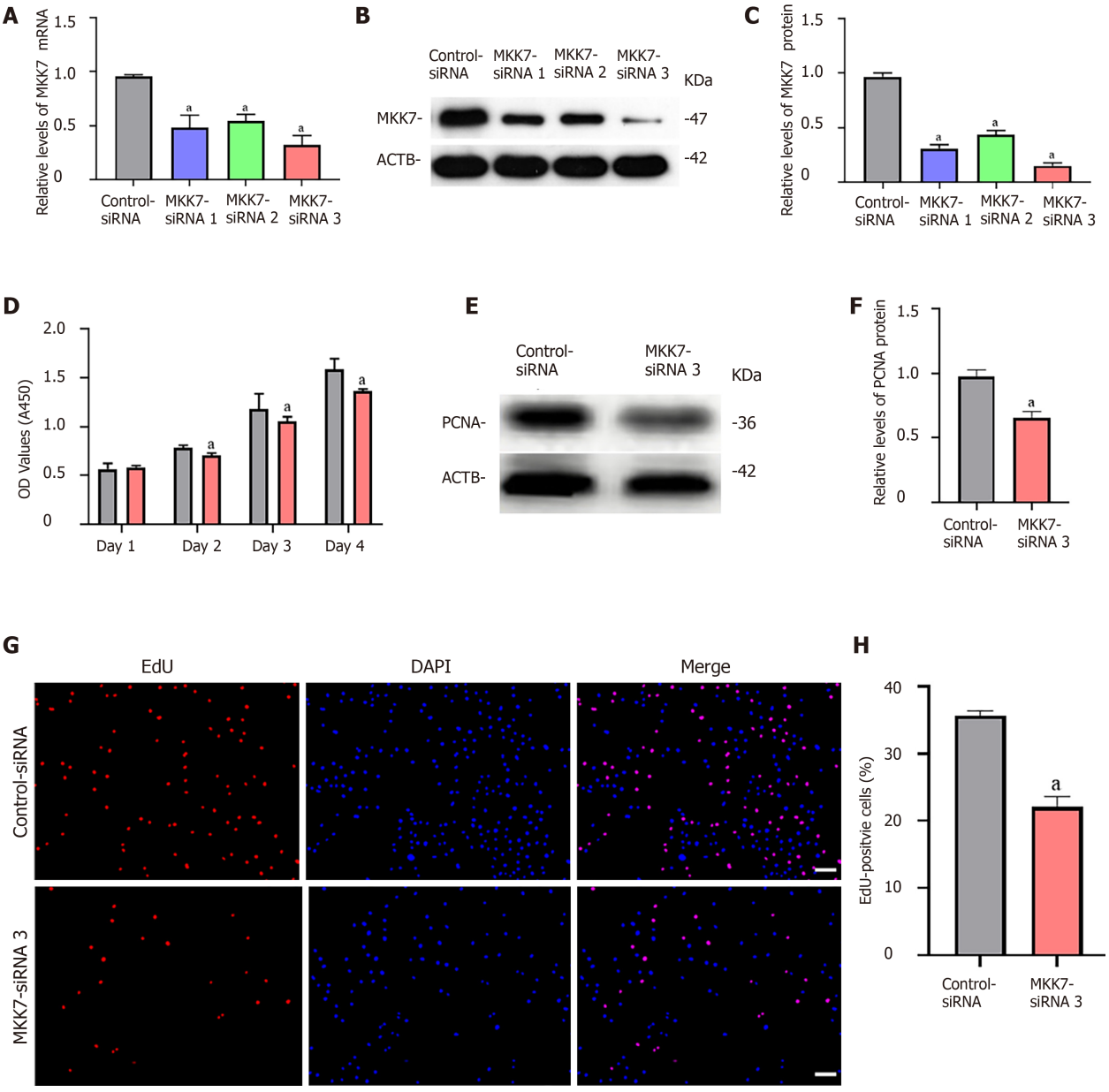

Figure 2 The influence of mitogen-activated protein kinase kinase 7 knockdown on human spermatogonial stem cell proliferation. A: Quantitative real time PCR revealed mitogen-activated protein kinase kinase 7 (*MKK7*) mRNA level changes in a human spermatogonial stem cell (SSC) line after treatment with MKK7-small interfering RNA (siRNA) 1-, 2- and 3; B and C: Western blotting showed MKK7 protein level alterations in the human SSC line after the transfection of *MKK7*-siRNA 1-, 2- and 3. Beta actin (ACTB) was used as the loading control for the total protein; D: Cell counting kit (CCK)-8 assay illustrated the proliferation of human SSCs transfected with the Control-siRNA and *MKK7*-siRNA 3; E and F: The relative levels of proliferating cell nuclear antigen (PCNA) protein in human SSCs after transfection with the Control-siRNA and *MKK7*-siRNA 3; G and H: The percentages of EdU-positive cells in human SSCs transfected with the Control-siRNA and *MKK7*-siRNA 3. Scale bars: G, 50 μm. $^{a}P < 0.05$ denotes a significant difference between the *MKK7*-siRNA 3 and Control-siRNA groups. DAPI: 4,6-diamidino-2-phenylindole.

TUNEL positive cells increased after *MKK7* silencing compared with that in the control siRNAtransfected cells (14.34% ± 1.83% *vs* 6.01% ± 0.95%, $P < 0.05$) (Figure 3D and E). Taken together, the results suggested that MKK7 promotes DNA synthesis and cell proliferation, whereas *MKK7* knockdown causes apoptosis.

MKK7 promotes the phosphorylation of JNKs

We further investigated the targets of MKK7. We predicted the targets of MKK7 using bioinformatic analysis, (GeneMANIA, HitPredict and String). Comprehensive prediction using the three databases identified JNK1, JNK2 and mitogen-activated protein kinase kinase kinase 7 as MKK7interacting proteins (Figure 4A). Mitogen-activated protein kinase kinase kinase 7 was reported as an upstream activator of the MKK/JNK signal transduction pathway, acting *via* phosphorylation of MKK7[32], and MKK7 was reported to activate JNKs by phosphorylation[26]; therefore, JNKs might

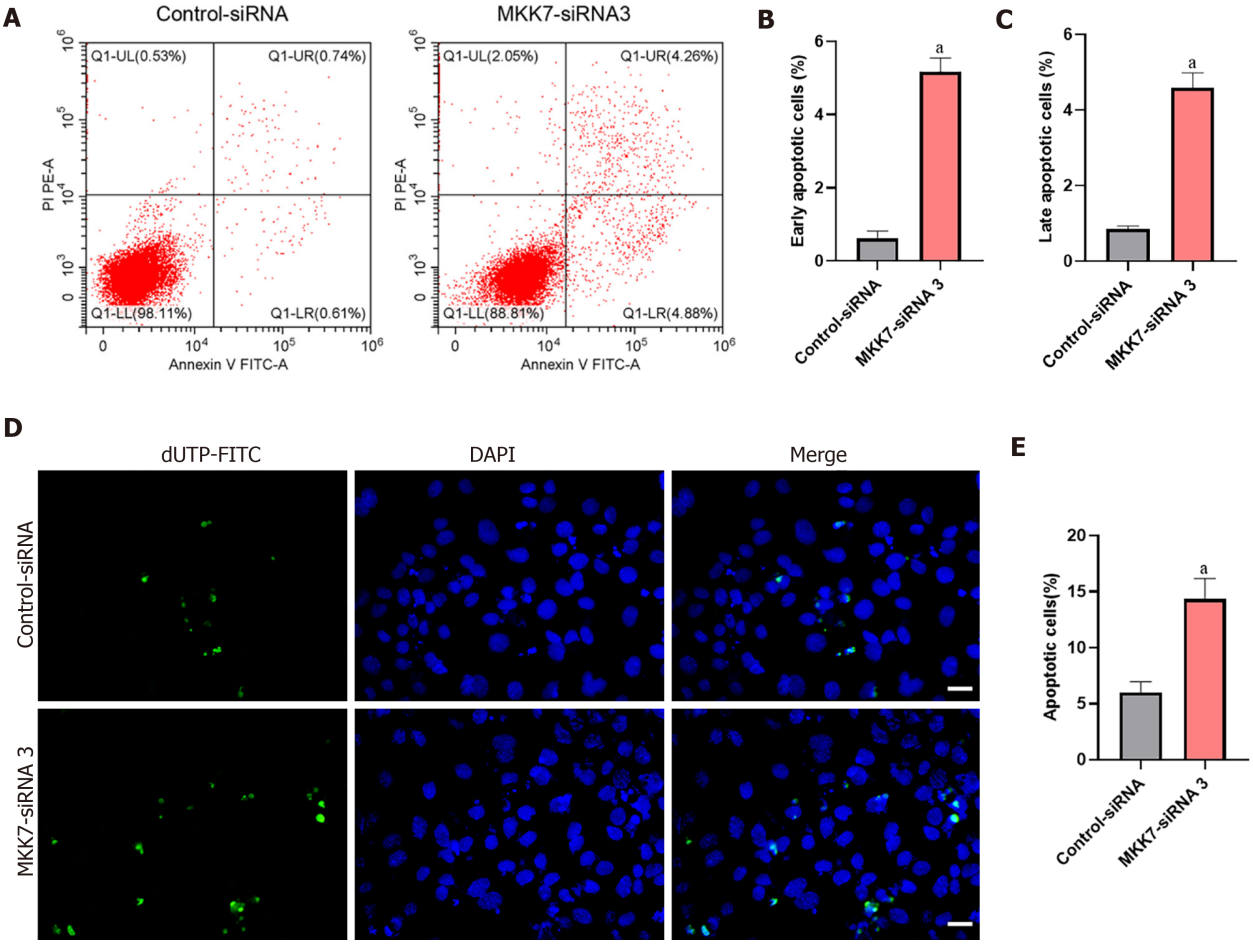

Figure 3 The influence of mitogen-activated protein kinase kinase 7 knockdown on the apoptosis of human spermatogonial stem cells. A-C: Flow cytometry and fluorescein isothiocyanate (FITC) Annexin V analysis of the percentages of early (A and B) and late (A and C) apoptosis cells in human spermatogonial stem cells (SSCs) transfected with the Control-small interfering RNA (siRNA) and mitogen-activated protein kinase kinase 7 (MKK7)siRNA 3; D and E: Terminal deoxynucleotidyl transferase nick-end-labeling (TUNEL) assays of the percentages of TUNEL$^+$ cells in human spermatogonial stem cells transfected with the Control-siRNA and MKK7-siRNA 3. Scale bars in D: 50 μm. $^aP < 0.05$ denotes a significant difference between the MKK7-siRNA 3 and Control-siRNA groups. dUTP: Deoxyuridine 5'-triphosphate; DAPI: 4,6-diamidino-2-phenylindole.

be candidate downstream targets of MKK7 in SSCs. To examine whether there was a physical interaction between MKK7 and JNKs, an immunoprecipitation assay followed by western blotting was carried out (Figure 4B), which showed that MKK7 could bind directly to JNKs in the human SSC line. By contrast, the control IgG did not pull down JNKs. We further detected the levels of JNKs and phosphorylated JNKs in human SSCs transfected with *MKK7*-siRNA3 (Figure 4C). We found that the levels of phosphorylated JNKs were reduced significantly compared with those in cells transfected with the control siRNA; however, the total level of JNKs did not change significantly. This result indicated that MKK7 could bind directly to JNKs to promote their phosphorylation.

Inhibition of JNKs decreases proliferation and promotes apoptosis of human SSC

To verify whether MKK7 affects SSC proliferation through phosphorylation of JNKs, we blocked the function of JNKs using SP600125, a selective inhibitor of JNK phosphorylation. We detected the level of phosphorylated JNKs after SP600125 treatment for 24 h, which showed that the levels of phosphorylated JNKs were notably reduced compared with those in the untreated control group (Figure 5A and B). In addition, PCNA protein levels decreased significantly (Figure 5C and D). Then, we examined the proliferation of human SSCs using the CCK-8 assay. SP600125 treatment significantly inhibited cell proliferation compared with that in the control group (Figure 5E). In addition, the proportion of EdU-positive cells decreased after culture with SP600125 for 24 h (34.17% ± 1.56% *vs* 21.84% ± 1.62%, $P < 0.05$) (Figure 5F and G). We further analyzed the apoptosis of human SSCs using the TUNEL assay, which showed that TUNEL positive cells increased significantly (5.13% ± 0.34% *vs* 12.18% ± 1.03%, $P < 0.05$) after SP600125 treatment (Figure 5H and I). Likewise, SP600125

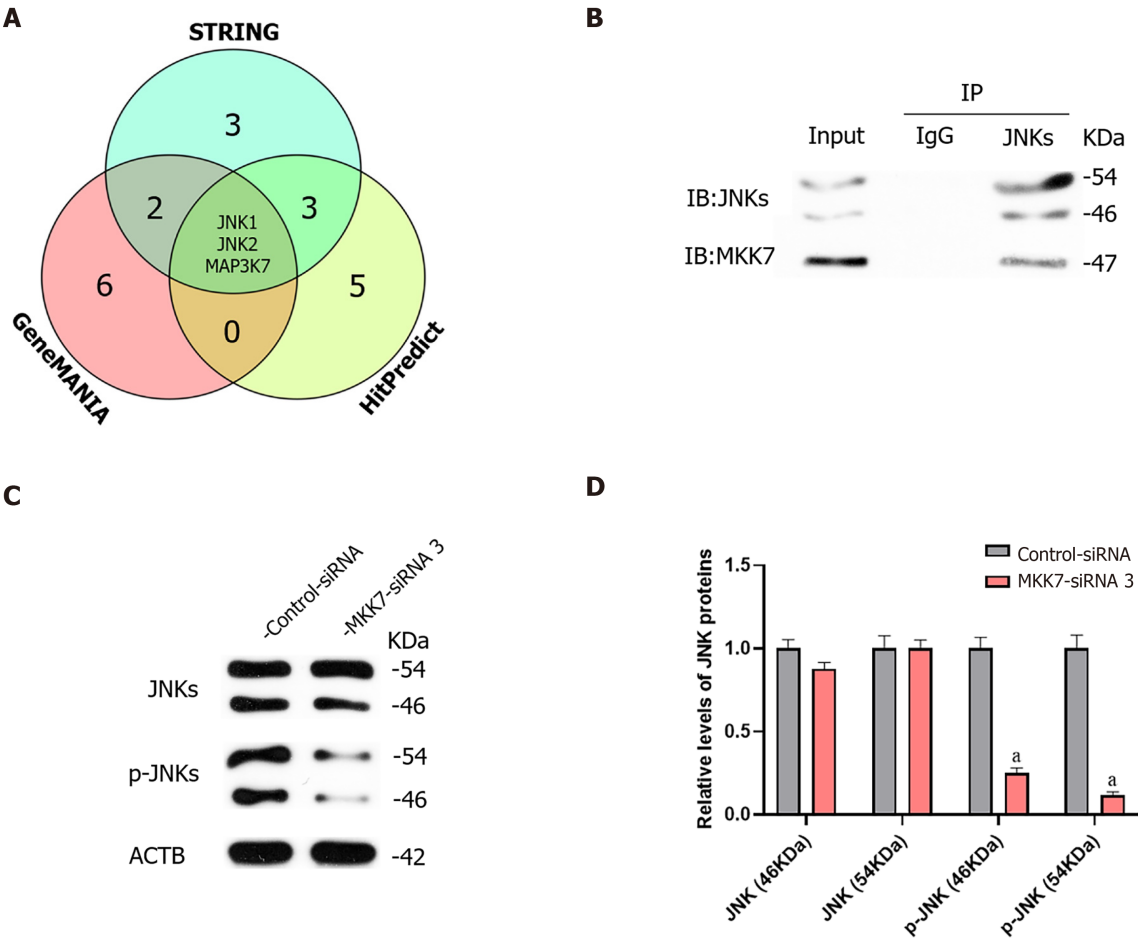

Figure 4 Mitogen-activated protein kinase kinase 7 affected the phosphorylation of c-Jun N-terminal kinases. A: Three databases predicted that mitogen-activated protein kinase kinase 7 (MKK7) interacts directly with c-Jun N-terminal kinase 1 (JNK1), JNK2 and mitogen-activated protein kinase kinase kinase 7 (MAPK3K7); B: Protein co-immunoprecipitation (IP) results indicated that MKK7 interacts directly with JNKs; C and D: Western blotting results showed that *MKK7* knockdown did not affect the overall expression level of JNKs; the levels of phosphorylated (p-)JNKs decreased significantly. $^{a}P < 0.05$ denotes a significant difference between the *MKK7*-small interfering (si)RNA 3 and Control-siRNA groups. IB: Immunoblot; IgG: Immunoglobulin G; ACTB: Beta actin.

treatment caused an increase in early apoptosis but not late apoptosis of SSCs according to Annexin V/propidium iodide staining and flow cytometry [1.80% ± 0.25% (control) *vs* 4.61% ± 0.45% (SP600125), $P < 0.05$] (Figure 5J-L). Taken together, the results indicated that SP600125-mediated inhibition of JNK phosphorylation in SSCs resulted in impaired cell proliferation and increased cell apoptosis.

MKK7 downregulation correlates with NOA

NOA is a serious male infertility disease in clinical practice. According to the results of testicular pathological tissue, NOA can be divided into spermatogonia maturation arrest, spermatocyte maturation arrest, spermatid maturation arrest, hypospermatogenesis and Sertoli cell only syndrome. To investigate whether MKK7 is associated with impaired spermatogenesis in adults, we examined MKK7 expression patterns in eight testes. According to the results of hematoxylin-eosin staining, we confirmed the spermatogenesis status of the testis (Supplementary Figure 2A-H). We examined the positive proportion and localization changes of MKK7 (red) in SSCs using double immunohistochemistry with ubiquitin C-terminal hydrolase L1 (green). The results indicated that the percentage of MKK7-expressing SSC was significantly reduced in spermatogonia maturation arrest and spermatocyte maturation arrest samples compared to samples with normal spermatogenesis (Figure 6A and B), and the fluorescence intensity appeared to decrease in hypospermatogenesis samples, but there were no translocations of MKK7 protein observed in NOA samples. We further detected the relative levels of MKK7 protein by using western blots. The results displayed that the expression of MKK7 was significantly downregulated in all samples with impaired spermatogenesis compared to the normal group (Figure 6C and D). Those results implied that the aberrant expression of MKK7 was correlated with spermatogenesis disorder, especially the maturation of spermatogonia and sperma-

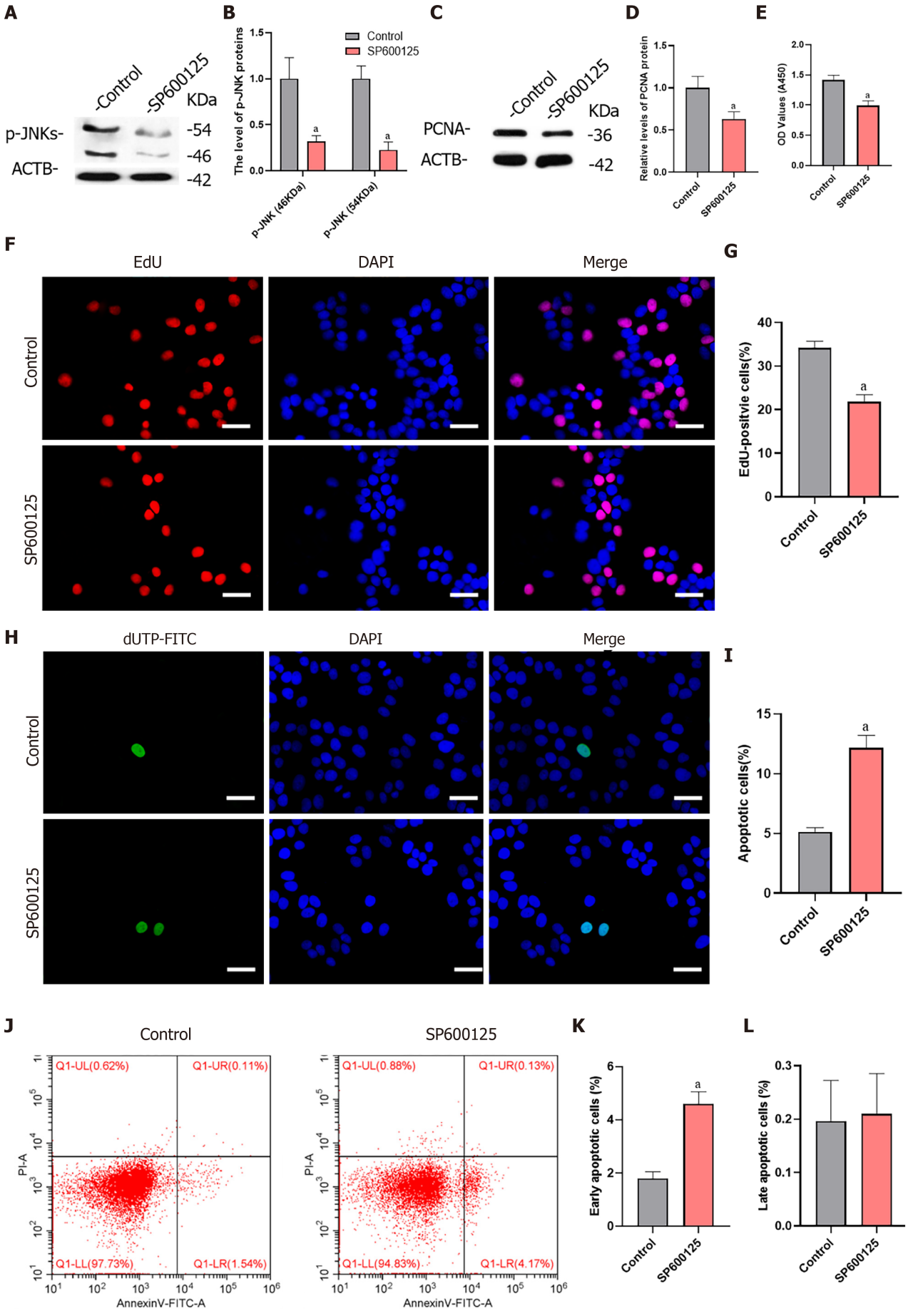

Figure 5 Inhibition of c-Jun N-terminal kinase 1 phosphorylation inhibited proliferation and promoted apoptosis of human

spermatogonial stem cells. A and B: Western blotting results showed that the phosphorylation of c-Jun N-terminal kinases was significantly inhibited by the c-Jun N-terminal kinase phosphorylation inhibitor, SP600125; C and D: The levels of proliferating cell nuclear antigen (PCNA) protein in human spermatogonial stem cells (SSCs) decreased significantly after treatment with SP600125; E: Cell counting kit (CCK)-8 results showed that the proliferation of SSCs was downregulated; F and G: EdU assays of DNA synthesis in human SSCs treated with SP600125; H and I: Terminal deoxynucleotidyl transferase nick-end-labeling (TUNEL) assay of the percentage of TUNEL+ cells in the human SSC line after SP600125 treatment; J-L: Flow cytometry and fluorescein isothiocyanate (FITC)/Annexin V analysis of the proportions of early (J and K) and late (J and L) apoptosis in the human SSC line treated with SP600125. Scale bars in F and H: 50 μm. $^{a}P < 0.05$ denotes a significant difference between the Control and SP600125 treatment groups. p-JNK: Phosphorylated c-Jun N-terminal kinase; ACTB: Beta actin; DAPI: 4,6-diamidino-2-phenylindole.

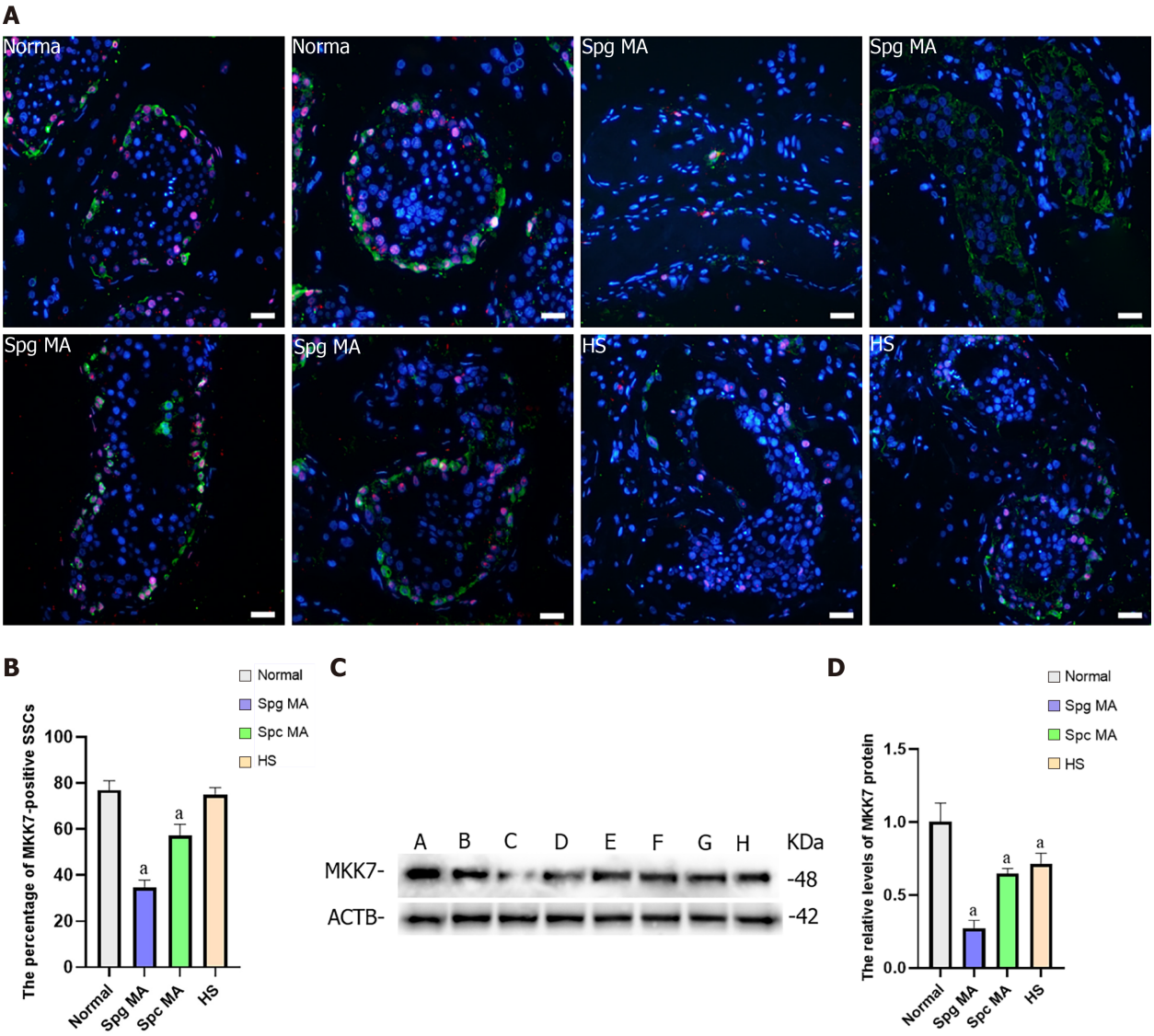

Figure 6 The expression of mitogen-activated protein kinase kinase 7 in the testis of obstructive azoospermia patients and non-obstructive azoospermia patients. A and B: The percentages of ubiquitin C-terminal hydrolase L1 (UCHL1) (green)-positive spermatogonial stem cells (SSCs) with mitogen-activated protein kinase kinase 7 (MKK7) (red) expression between obstructive azoospermia (OA) and various kinds of non-obstructive azoospermia (NOA) patients; C and D: Western blotting compared the relative levels of MKK7 protein between OA and NOA patients. Notes in (C): sample A and B were individuals with OA with normal spermatogenesis; sample C and D were NOA patients with spermatogonia maturation arrest; sample E and F were NOA patients with spermatocyte maturation arrest; sample G and H were NOA patients with hypospermatogenesis. Scale bars: C, 50 μm. $^{a}P < 0.05$ indicated the significant differences between OA patients with normal spermatogenesis and NOA patients. Spg MA: Spermatogonia maturation arrest; Spc MA: Spermatocyte maturation arrest; HS: Hypospermatogenesis; ACTB: Beta actin.

tocytes.

DISCUSSION

Human SSCs are the origin of spermatogenesis[33]; however, research on human SSCs are hampered because of limited sources and scarce numbers[34,35]. Much is known about the mechanism of SSC regulation in rodents, and spermatogenesis has been reconstructed through SSC transplantation[36]. However, these results are not entirely applicable to primates because many biological processes of rodent SSCs are different from those of primates[15,37]. For example, the spermatogenic epithelial cycle in mice can usually be divided into 12 stages; however, humans generally have 6 stages[21, 22]. Furthermore, octamer-binding protein 4, a biomarker of mouse SSCs, is not expressed in human SSCs[23,24]. Therefore, research on the mechanisms of human SSCs are hindered by these discrepancies. Human primary SSCs cannot be cultured *in vitro* for a long time because of the weak proliferation of human SSC *in vitro*, and the culture system of mice SSCs is not suitable for human SSC. In addition, the proliferation of human SSCs *in vitro* is weak[34]. To establish the human SSC line, the human SV40 Large T antigen was overexpressed in human primary GPR125-positive undifferentiated spermatogonia[29,38]. These cells possess similar biological properties to human SSCs and possess an unlimited proliferative capacity, which assisted us in studying the molecular mechanisms of SSCs.

MKK7 is involved in the phosphorylation of JNKs and activates their downstream pathways[39]. However, the promotion of SSC proliferation by MKK7 was unexpected, given that its inhibition often enhances self-renewal division of other stem cells. In mouse embryonic stem cells, activation of c-Jun NH2-terminal kinase by $CdCl_2$ - or $HgCl_2$ requires MKK7[40], and it is also involved in the process of embryonic stem cell differentiation into cardiomyocytes[41]. This suggested that MKK7 regulation in SSCs is distinct from that in other stem cells. In our study, we found that MKK7 was primarily expressed in self-renewing SSCs ($GFRA1^+/PCNA^+$), which was consistent with the findings of single cell sequencing, in which MKK7 was mainly expressed in SSCs[42]. Therefore, we believe it may be associated with the proliferation or apoptosis of SSCs. We validated the functions of MKK7 in a human SSC cell line using siRNA, and the results showed that MKK7 deficiency inhibited proliferation and promoted apoptosis of SSCs. MKK7 has been reported to be involved in the apoptotic process in neural cells[43] and participates in the proliferation of hepatocytes and cancer cells[44, 45]. The functional diversity of MKK7 in various cells might reflect different downstream targets. Our work provides more evidence for the functions of MKK7; however, the specific downstream effectors of MKK7 remain to be explored.

Previous reports and bioinformatic prediction suggested that MKK7 is involved in the phosphorylation modification of JNKs[39] and blocking JNKs using small molecule inhibitors similarly inhibited the proliferation of mouse SSCs[28]. The development of SSCs is regulated by many key growth factors, such as GDNF[46], FGF[12], epidermal growth factor[47] and LIF interleukin 6 family cytokine[8]. Other intracellular regulatory molecules, including promyelocytic leukemia zinc finger[48], Nanos C2HC-type zinc finger 2[49] and Spalt like transcription factor 4[50], have also been demonstrated to be involved in SSC fate determination. Although the phosphorylation of JNK1 and JNK2 were reported to increase after treatment with GDNF and FGF2 in mouse SSCs[28], whether MKK7 is involved in the signal transduction of these important regulatory factors or if other regulatory pathways participate in this process remains to be further investigated. It is also reported that cell division cycle 5 like[51] and TNF receptor associated factor 6[52] have direct interactions with MKK7, which suggests that MKK7 might also affect cell proliferation through other molecules. To explore this question, further investigations are needed, for example, protein co-immunoprecipitation combined with mass spectrometry and transcriptome sequencing.

Although the pathogenesis of NOA is largely unclear, our results displayed that MKK7 levels were reduced in the testis of patients with NOA compared with those in patients with obstructive azoospermia, especially in testis with spermatocyte maturation arrest and spermatogonia maturation arrest. A reduction was also observed in the proportion of MKK7-positive SSCs. Exploration of mutations in the *MKK7* gene in patients with NOA and the construction of point mutations or knockout mouse models should be used to confirm the functions of MKK7. In addition, the current study was carried out in a human SSC line, and the proliferation function of MKK7 was only tested in SSCs. Thus, we are unsure whether MKK7 is involved in the differentiation of progenitor cells, which also needs further studies in mouse models.

In conclusion, we revealed that MKK7 is expressed mainly in human SSCs and inhibits the apoptosis and enhances the proliferation of SSCs *via* JNK phosphorylation. Although some questions remain, our study offers new clues regarding the pathways

and genes involved in the determination of SSC fate in humans. The activation of MKK7 or JNK1 using small molecules might contribute to human SSC self-renewal *in vitro* and might identify molecular targets to diagnose and treat male infertility.

CONCLUSION

MKK7 regulates the proliferation and apoptosis of human SSC by mediating the phosphorylation of JNKs. Abnormal expression of MKK7 may impair human spermatogenesis.

ARTICLE HIGHLIGHTS

Research background

Human spermatogonial stem cells (SSCs) are the basis of spermatogenesis. However, little is known about the developmental regulatory mechanisms of SSC due to sample origin and species differences.

Research motivation

To investigates the mechanisms involved in the proliferation of human SSCs.

Research objectives

To investigate the functions and mechanisms of mitogen-activated protein kinase kinase 7 (MKK7) during proliferation and apoptosis in human SSCs.

Research methods

The expression of MKK7 in human testis was identified using immunohistochemistry and western blotting (WB). MKK7 was knocked down using small interfering RNA, and cell proliferation and apoptosis were detected by WB, EdU, cell counting kit-8 and fluorescence-activated cell sorting. After bioinformatic analysis, the interaction of MKK7 with c-Jun N-terminal kinases (JNKs) was verified by protein co-immunoprecipitation and WB. The phosphorylation of JNKs was inhibited by SP600125, and the phenotypic changes were detected by WB, cell counting kit-8 and fluorescence-activated cell sorting.

Research results

MKK7 is mainly expressed in human SSCs, and MKK7 knockdown inhibits SSC proliferation and promotes their apoptosis. MKK7 mediated the phosphorylation of JNKs, and after inhibiting the phosphorylation of JNKs, the phenotypic changes of the cells were similar to those after MKK7 downregulation. The expression of MKK7 was significantly downregulated in patients with abnormal spermatogenesis, suggesting that abnormal MKK7 may be associated with spermatogenesis impairment.

Research conclusions

MKK7 regulates the proliferation and apoptosis of human SSC by mediating the phosphorylation of JNKs. Abnormal expression of MKK7 may impair human spermatogenesis.

Research perspectives

This study intended to reveal the role and regulatory mechanism of MKK7 in the regulation of SSC development and spermatogenesis in humans, which can provide a scientific basis for the etiological interpretation and molecular diagnosis of male infertility. It also provided new molecular targets for the clinical treatment of male infertility and the development of contraceptives.

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