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ABOUT COVER

Editorial board member of *World Journal of Stem Cells*, Dr. José Bragança is Professor at the University of Algarve, Portugal. Having received his Bachelor's and Master's degrees in Biochemistry from the University Paris VI, he then obtained a PhD in Biochemistry and Molecular Biology at the Université Paris XI in 1998. He held a post-doctoral position in the Department of Cardiovascular Medicine at the University of Oxford (1999-2007), before moving to Portugal to establish his research group. His ongoing research interests involve the study of the molecular mechanisms important for the establishment and the maintenance of pluripotency of stem cells. Currently, he is a member of the Directive Board of the Algarve Biomedical Centre and Vice-President of the Portuguese Society for Stem Cells and Cell Therapies.

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REVIEW

Potential of transposon-mediated cellular reprogramming towards cell-based therapies

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Abstract

Induced pluripotent stem (iPS) cells present a seminal discovery in cell biology and promise to support innovative treatments of so far incurable diseases. To translate iPS technology into clinical trials, the safety and stability of these reprogrammed cells needs to be shown. In recent years, different non-viral transposon systems have been developed for the induction of cellular pluripotency, and for the directed differentiation into desired cell types. In this review, we summarize the current state of the art of different transposon systems in iPS-based cell therapies.

Key Words: Transposons; Induced pluripotent stem cells; Clinical applications; Cellular reprogramming; Cell-based therapy; Genetic correction

Core tip: The seminal discovery of induced pluripotent stem (iPS) cells has opened up the possibility of converting most somatic cell types into a pluripotent state. The iPS cells possess most of the advantages of embryonic stem cells without the ethical stigma associated with derivation of the latter. This procedure has had a large impact on the generation of custom-made pluripotent cells, ideal for cell-type specific differentiation and regenerative medicine with or without genetic correction. In this review, we focus on updated information of transposon system-mediated cellular reprogramming to iPS cells

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INTRODUCTION

Transposon systems currently provide a promising toolbox for cell therapy, disease modeling, and drug discovery[1-4]. Importantly, the non-viral transposon systems can be an important alternative to viral vectors, which are commonly used for cellular reprogramming for transfection of somatic cells with exogenous *Oct4*, *Sox2*, *Klf4*, and *c-Myc* genes to induce cellular pluripotency and establish induced pluripotent stem (iPS) cells[5-8]. However, the limited cargo size of retro and lenti viral vectors of about 7 kb pairs hampers transfer of larger therapeutic genes[9]. In addition, the construction of viral vectors is cumbersome, expensive and requires living cells for their scale up, which further complicates the quality control and downstream processing[10].

The iPS cell technology promises to provide an unlimited source of cells for innovative therapies, and to treat so far incurable diseases[11-13]. A hypothetical schedule would require a small tissue sample from the patient, to reprogram the somatic cells to iPS cells with unlimited proliferative capacity, to perform gene correction in the iPS cells, then to direct differentiation into the desired precursor cells, which are finally transplanted into the patient (Figure 1).

In this respect, Sleeping Beauty (SB) and piggyBac (PB) transposon systems appear as attractive tools for somatic cell reprogramming due to their efficient gene delivery and their ability to be excised from the cells after reprogramming, which helps overcome the limitations of viral-based reprogramming technologies. Transposon systems have a number of additional advantages, such as (1) Cargo capacity of up to 100 kb[14,15]; (2) No bias to integrate in expressed genes or promoter regions; (3) Possibility of seamless removal of the transposon[16,17]; (4) Cost-effective production of the basic plasmids; (5) Reduced innate immunogenicity; and (6) No requirement for a specialized biosafety facility.

The translation of this iPS cell-based therapy into clinical testing needs authorization approval to initiate safety and efficacy studies, and to exclude risks of insertional oncogenesis or immunogenicity[18,19]. SB and PB transposon systems have been successfully used to obtain reprogrammed iPS cells from human somatic cells[16,20], but also somatic cells from the murine model[21-24], and cells from large model species, such as pig[25], horse[26], bat[27], monkey[28], rat[29], cattle[30,31] and buffalo[32]. Here, we review the potential of transposon-mediated cellular reprogramming and its clinical applications in cell-based therapy and the associated risks.

SHORT SYNOPSIS OF THE MOST COMMONLY APPLIED TRANSPOSON SYSTEMS

DNA transposons, also known as Class II elements or mobile genetic elements, were first described as "jumping genes" by McClintock[33] and were found to be responsible for color mosaicism of maize cob kernels. DNA transposons have been divided into two major groups: (1) Cut-and-paste; and (2) Rolling-circle transposons[34]. In vertebrates, commonly cut-and-paste group of transposons are found, which include the Tc1/mariner, hATs, PB and SB families, all of which are characterized by inverted terminal repeats of 10 to 1000 bp flanking their transposase gene[35]. Transposons are discrete DNA segments which can move from one site to another within a genome, and sometimes between genomes catalyzed by the transposase[36,37]. Transposons are species-specific, found in the genomes of all prokaryotes and eukaryotes, whereas in humans approximately 46% of the genome is derived from retro- (RNA) and DNA transposons[38,39].

Transposons are important sources of genome structures that are actively used to regulate the multicellular embryonic development. These structures include binding

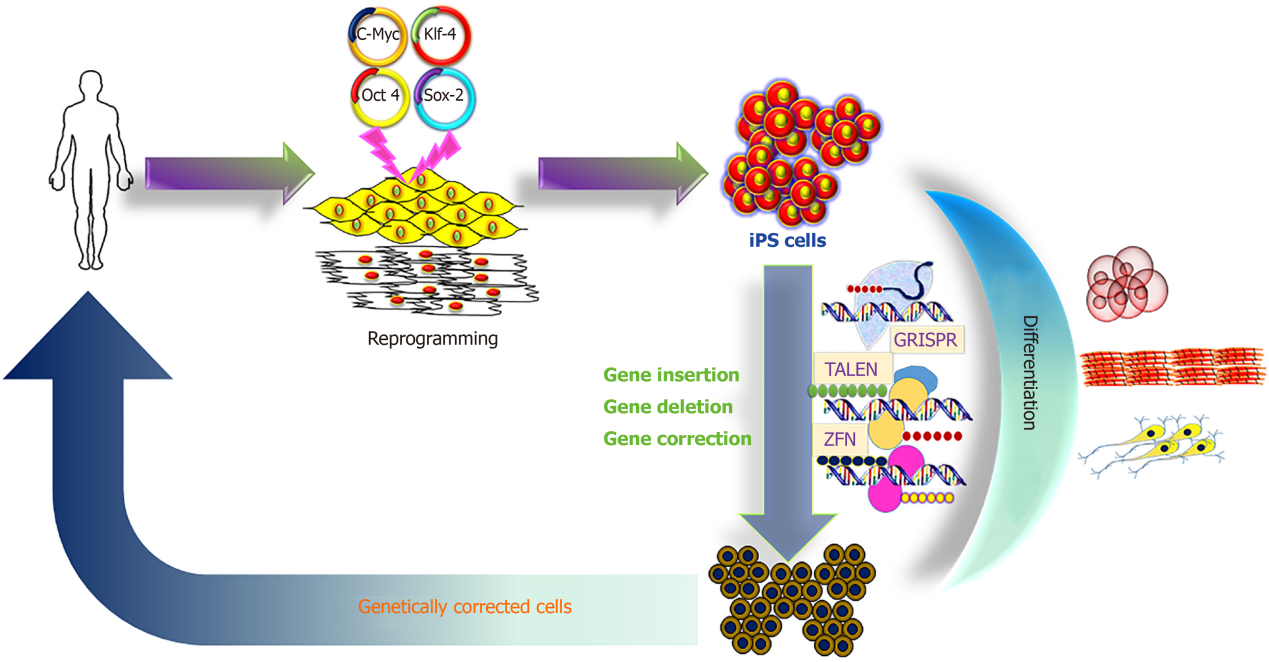

Figure 1 Schematic representation of induced pluripotent stem cell derivation, differentiation and genetic modification. iPS: Induced pluripotent stem; CRISPR: Clustered regularly interspaced short palindromic repeats; TALEN: Transcription activator-like endonucleases; ZFN: Zinc finger nucleases.

sites with transcription factors, enhancers and silencers, promoters, insulators, alternative splicing sites, and non-coding RNA. Moreover, transposons are involved in the emergence and evolution of new protein-coding genes through exonization, domestication, and the formation of retrogenes. The activation of transposons is needed to regulate the differentiation and reproduction of cells in the body; however, in terminally differentiated cells, upon reaching predetermined sizes of organs, molecular systems are activated that block a further cascade of transposon activation[40,41]. Due to the wide distribution and diversity of transposons, they contribute significantly to genomic variation and as such, they are powerful drivers of genome evolution[36,42-45].

For this purpose, SB and PB transposon systems are identified as efficient vectors for cellular reprogramming. The SB originated from salmonid fish species, where it existed as an inactive element[46]; from this a synthetic transposon system was constructed using a reverse engineering approach to eliminate the accumulated mutations[46]. PB was derived from an active element discovered in the moth *Trichoplusia ni*[47]. These transposons have no orthologous elements in mammalian species, which prevents the re-mobilization of transposons by potential endogenous transposases. This has been experimentally verified in transgenic mice and pigs[48,49]. Presently, the hyperactive versions of SB (SB100X) or PB (hypPB) seem to be the most active transposon systems. They possess comparable activity levels in mammalian cells, and are independent of cellular co-factors[50,51]. Both of these transposons have been employed for stable expression of reprogramming factors and are suitable for the derivation of iPS cells as proven in various studies[16,22,23,25,26,30-32,52]. Other transposons namely: Frog Prince, Mos1, Tol2 and Passport are also active in mammalian cells, but they are still under-investigated in iPS cell generation[53].

MECHANISM OF TRANSPOSON-MEDIATED CELLULAR RE-PROGRAMMING

The recombinant PB and SB systems mobilize or transfer gene(s) of interest through a "cut and paste" mechanism (Figure 2)[2,54,55]. For most applications, recombinant transposon systems encompass a donor plasmid that carries one or more genes flanked by the inverted terminal repeats (ITRs) sequences essential for transposition[2,56,57]. The transposase gene can be positioned on a separate plasmid (trans) or in the same plasmid (cis). Once the transposase protein is expressed, it binds

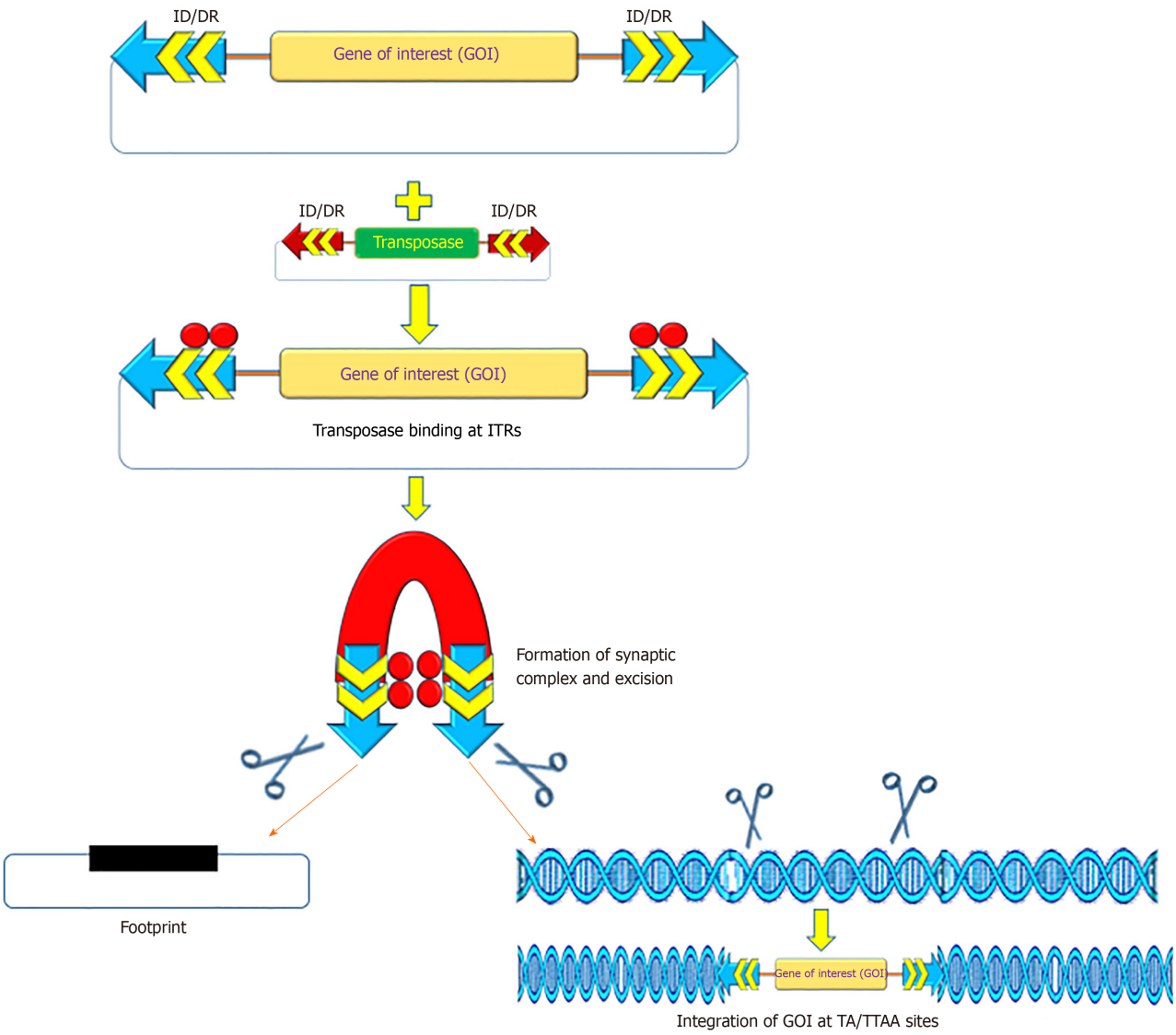

Figure 2 Mechanism of action of transposon-transposase mediated transposition. ITRs: Inverted terminal repeats; IR: Inverted repeats; DR: Direct repeats.

to the ITR sequences, which catalyzes the removal of the gene of interest (cut) and integrates (paste) the transposon sequence into the genome of a host cell[57]. The SB transposase catalyzes integrations at consensus TA-dinucleotides[46], whereas the PB requires TTAA-tetranucleotide sequences[58-60]. The efficiency of transposition of these transposon systems has been further increased due to generation of highly active and efficient transposases, namely hyp(er) PB (hypPB) and hyperactive SB 100X (hySB100X)[50,51,61,62]. The hySB100X showed a 30% higher transposition rate compared with SB100X. hySB100X was obtained by mutation in short hydrophilic residues in the catalytic domain of the SB100X transposase molecule, which required direct DNA contact to increase the DNA binding affinity of the transposon[62]. Furthermore, the transposition rate of these transposons is affected by topological conformations, chromatin condensation and CpG-methylation patterns of the target DNA[63,64]. Genomic insertion for SB100X prefers target regions with higher AT content, in a palindromic core unit[65,66]; whereas PB transposase integration requires a TTAA recognition sequence and exhibits a bias toward insertions in genes[67].

For cellular reprogramming, the transfection of the transcription factors into somatic cells using the transposon system is relatively straightforward. The transposons-mediated cellular reprogramming leads to an overall efficiency of approximately 0.02%[20,22,23,30], which nears the initially obtained reprogramming efficiencies by viral vectors. The obtained reprogramming efficiency from transposons is higher than other reported non-integrative delivery systems including either replicating episomal vectors or minicircles[68,69], although lower than Sendai viral vectors or synthetic

mRNA[70,71]. Transposons-mediated transposition is a self-regulated activity *via* overproduction inhibition, a mechanism by which transposition activity is down-regulated when the transposase is over concentrated in cells[72]. Ideally, the transposase is expressed only for a short period, which prevents continuous transposon re-mobilization. However, it is also important to minimize the number of vector copies per cell as it poses an increased risk of insertional oncogenesis[73].

THE EXPANDING TRANSPOSON TOOLBOX

Transposon systems are widely used for gene delivery applications[58,74-76]. However, like the lenti viruses, transposon vectors are mutagenic, because of their random integration. Recently, clustered regularly interspaced short palindromic repeats (CRISPR) and Cas9 nucleases have emerged as excellent tools for site-specific mutation of genomes[77]. This system is an attractive candidate for targeting through extensive base pairing with the target[78]. In contrast, most DNA binding proteins remain bound to their target sites only for a matter of seconds or minutes. However, double-stranded breaks induced by CRISPR-Cas9 nucleases showed undesirable outcomes in terms of large deletions extending over many kilobases at high frequency and complex genomic rearrangements[79]. To overcome the challenges of nuclease-based gene delivery, various research groups have attempted to use site-specific DNA binding proteins such as SB, PB, Mos1, and ISY100-fused with zinc finger protein, transcription activator like effector (TALE) and/or Gal4 to target specific loci[80-82]. Owens *et al*[83] fused a TALE DNA-binding domain (DBD) with PB to direct the transposase to stimulate insertional activity of PB at the intended target sequence. This approach allowed the isolation of clones harboring single-copy insertions at the CCR5 locus. Subsequently, attempts were made using catalytically dead Cas9 (dCas9) for targeting PB insertions to the human endogenous hypoxanthine phosphoribosyl transferase (HPRT) locus[82]. Surprisingly, the dCas9-PB chimera protected it from insertions instead of targeting the HPRT locus. Although, PB is considered to be the most efficient system for gene delivery *in vivo*[84,85], it impedes the development of advanced applications such as direct delivery of transposons[86]. To resolve this difficulty, Chen and Wang described a Cas-Transposon (CasTn) system for genomic insertions which uses a Himar1 transposase fused with a dCas9 nuclease to mediate programmable, site-directed transposition[87]. They demonstrated that the Himar–dCas9 fusion protein improved the frequency of transposon insertion at a single targeted TA dinucleotide by > 300-fold compared to the un-fused transposase. This work highlights CasTn as a new modality for host-independent, programmable and site-directed DNA insertions[87].

More recently, Hew *et al*[88] tested a group of RNA-guided transposase vectors comprising mutations in the native PB DBD for their ability to target a single sequence in the *CCR5* gene. This RNA-guided transposition in human cells might be a framework for improved targeting vectors with potential applications in gene therapy and genome editing research[88]. Similarly, Stecker *et al*[89] found that the CRISPR-associated transposase derived from *Scytonema hofmanni* (ShCAST), catalyzes the site-specific RNA-directed unidirectional integration and is located a fixed distance to one side of the targeted DNA site. These sequence-specific integrations offer significant advantages over traditional virus-based integrating vectors by avoiding insertion into unwanted regions[90-93]. Another approach applied to generate "transient transgenesis" by mutation at position 248 in the SB transposase to gain further insight into the transposition mechanism and for the generation of reprogramming factor-free iPS cells[17]. The amino acid present at position 248 of the SB transposase is involved in an interaction with target DNA, and because of the absence of integration activity, transposon removal by these transposase mutants results in extra-chromosomal circles, thereby terminating the transposition reaction[17,94]. This indicates that by the switching of a single amino acid, the SB transposase has into efficient unidirectional removal ability with utility in cellular reprogramming. In addition, soluble variants of the SB protein have been developed by genetic engineering, which allows for more control over the exposure time[95]. These underlying genome engineering procedures will reduce costs and improve the safety of genome modifications.

TRANSPOSON-MEDIATED CELLULAR REPROGRAMMING

Commonly, somatic cells were reprogrammed to pluripotency by the exogenous introduction of transcription factors (Oct3/4, Sox2, Klf4 and c-Myc). The resulting iPS cells demonstrate the features of embryonic stem (ES) cells, including the ability to form chimeras and contribute to the germ line[5]. Thereafter, iPS cells were generated either by the protein transduction approach[96], or in combination with small chemical molecules[97] without genetic modification. These reprogramming approaches suffer from low efficiency and require complicated and prolonged cell culture conditions[96,97]. Furthermore, these approaches need either extraction of crude cell lysates of cells expressing defined reprogramming factors or preparation of a large amount of recombinant reprogramming transcription factors from bacteria, which may be contaminated with unknown detrimental genetic materials. Thus, the use of a suitable gene-delivery reprogramming approach is a critical step in the generation of iPS cells for basic and clinical research.

More recently, DNA transposons appeared as alternative tools for cellular reprogramming in a wide range of cell types, including fibroblasts using cocktails of transcription factors. This technique is straightforward, less time consuming and easy to handle as compared to viral vectors (Figure 3). In general, PB and SB systems have been used for iPS cells generation in a broad range of domesticated and farm animal species[16,20,22,23,25,30,32,98-101], in addition to human cells[102-105]. The generation of iPS cells from domesticated and companion animal species such as cattle, pig, horse and buffalo is critically important for the establishment of disease models and economically valuable for the production of medically useful substances, *e.g.*, enzymes and growth hormones, which are either absent or inadequate in patients suffering from specific genetic diseases. More importantly, either iPS cells or differentiated cells from iPS cells could be directly used for cellular therapies, drug screening, and disease modeling thus significantly decreasing the extent to which animals are used for research purposes[4,106-110].

In this direction, cellular reprogramming through transposon systems represents one of the unique features of the excision of gene expression cassettes from the iPS cell genome through re-expression of integration-deficient transposase variants. Alternatively, excision can be achieved by either clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 (CRISPR/Cas9) or Cre/loxP recombination technology[22,94]. Using these technologies enable the production of "transgene-free" iPS cells, which could be beneficial in minimizing the risk of reactivation of reprogramming factors leading to oncogenic potential[94]. Similarly, Woltjen *et al*[111] showed that PB-mediated transgene excision does not leave a genetic trace in the host genome, thus providing the feasibility of seamless modification for "genetically unmodified iPS cells" production.

DIFFERENTIATION POTENTIAL OF TRANSPOSON-MEDIATED IPS CELLS

Currently iPS cells are considered a valuable resource for studying medicine and regenerative biology due to their tremendous differentiation capacity into almost all cell types of the body. In principle, the differentiated cells derived from iPS cells should behave in the same way as their *in vivo* counterparts in terms of both molecular and functional aspects, but it remains a challenge to direct cell fate decisions under *in vitro* conditions towards specific cell types[112]. In general, differentiation comprises the conversion of an iPS cell to a more specialized cell type, involving a transition from proliferation to specialization. This involves successive alterations in cell morphology, membrane potential, metabolic activity and responsiveness to specific signals. Differentiation leads to acquiring specific functions of differentiated cells depending upon the tissue in which they will finally reside[113].

The transposon-mediated iPS cells can be differentiated *in vitro* in the absence of appropriate growth factor (LIF/bFGF) or feeder cells. Under the appropriate conditions, such as suspension culture, embryoid bodies (EBs) can be formed from iPS cells of almost all species, such as human[20], mouse[21,23], bat[27], monkey[28], prairie vole[114], horse[26], bovine[31], rat[29] and buffalo[32] with expression of lineage specific for endoderm, mesoderm and ectoderm (Table 1). Pluripotency is one of the defining features of iPS cells. Perhaps the most definitive test of pluripotency is the blastocyst complementation assay. The contribution of iPS cells to the resulting chimeras has been assessed to determine the differentiation capacity and germline contribution. True pluripotent murine iPS cells were generated using PB[115] and SB[21]. To the best of

Table 1 Differentiation potential of transposon-mediated induced pluripotent stem cells

Species	Cell type	Transposon system	Reprogramming factors	Differentiation		Characterization of lineage specific differentiated cells through		Chimera	Germline contribution	Ref.
				In vitro	*In vivo*	Histology	Expression of gene/protein			
Bat	Fetal fibroblasts	PB	Human OKSMNL + NR5A2, and bat-specific miR302/367	EBs	Teratoma	Yes	Yes	NA	NA	Mo *et al*[27], 2014
Buffalo	Fetal fibroblasts	PB	Human OSKMNL	EBs	NA	NA	Yes (ectoderm-NF-68 and Cytokeratin 8; mesoderm-MSX1 and endoderm-GATA4)	NA	NA	Kumar *et al*[32], 2019
Cattle	Fetal fibroblasts	PB SB	Human OSKMNL Murine OSKM	EBs	Teratoma	Yes	Yes (Ectoderm- β III-Tubulin, Nestin; Mesoderm-Vimentin and Endoderm-AFP, GATA, PAX6	NA	NA	Talluri *et al*[30], 2015
	Fetal fibroblasts	PB	Bovine OSKM	EBs	Teratoma	Yes	Yes (Ectoderm- β III-Tubulin; Mesoderm-α-SMA and endoderm-AFP)	NA	NA	Zhao *et al*[31], 2017
Horse	Fetal fibroblasts	PB	Murine OSKM	EBs	Teratoma	Yes	NA	NA	NA	Nagy *et al*[26], 2011
Human	Skin fibroblasts	PB	OSKML	EBs, Keratinocyte	Teratoma	NA	Yes (ectoderm-K14; mesoderm-desmin and endoderm-AFP)	Ethically not allowed	Ethically not allowed	Igawa *et al*[116], 2014
	Fetal fibroblasts	SB	Murine OSKM	EBs	NA	NA	Yes (Ectoderm- β III-Tubulin, Nestin; Mesoderm-Vimentin and Endoderm-AFP	Ethically not allowed	Ethically not allowed	Davis *et al*[20], 2013
Monkey	Skin fibroblasts	PB	Monkey OSKMNL	EBs	Teratoma	Yes	Yes (Ectoderm- β III-Tubulin; Mesoderm-SMA and Endoderm-AFP	NA	NA	Debowski *et al*[28], 2015
Mouse	Fetal fibroblasts	PB	Murine OSKM	EBs	Teratoma	Yes	NA	Yes	Yes	Yusa *et al*[115], 2009
	Fetal fibroblasts	SB	Murine OSKM	EBs	Teratoma	NA	Yes (cardiac-cTnT and desmin and neuronal-nestin and Tuj1)	Yes	Yes	Muenthaisong *et al*[21], 2012

Pig	Fetal fibroblasts	SB	Murine OSKM	Neuronal lineage	Teratoma	Yes	Yes	NA	NA	Kues *et al*[25], 2013
Prairie vole	Fetal fibroblasts	PB	Murine OSKMNL	EBs	Teratoma	Yes	NA	NA	NA	Katayama *et al*[114], 2016
Rat	Fetal fibroblasts	PB	OSKM	EBs, chondrocyte	NA	NA	NA	NA	NA	Ye *et al*[29], 2015

O: Oct4; S: Sox2; K: klf4; M: cMyc; N: Nanog; L: Lin28; EBs: Embryoid bodies; NA: Not applicable; PS: PiggyBac; SB: Sleeping beauty; AFP: α-Fetoprotein; SMA: Smooth muscle actin.

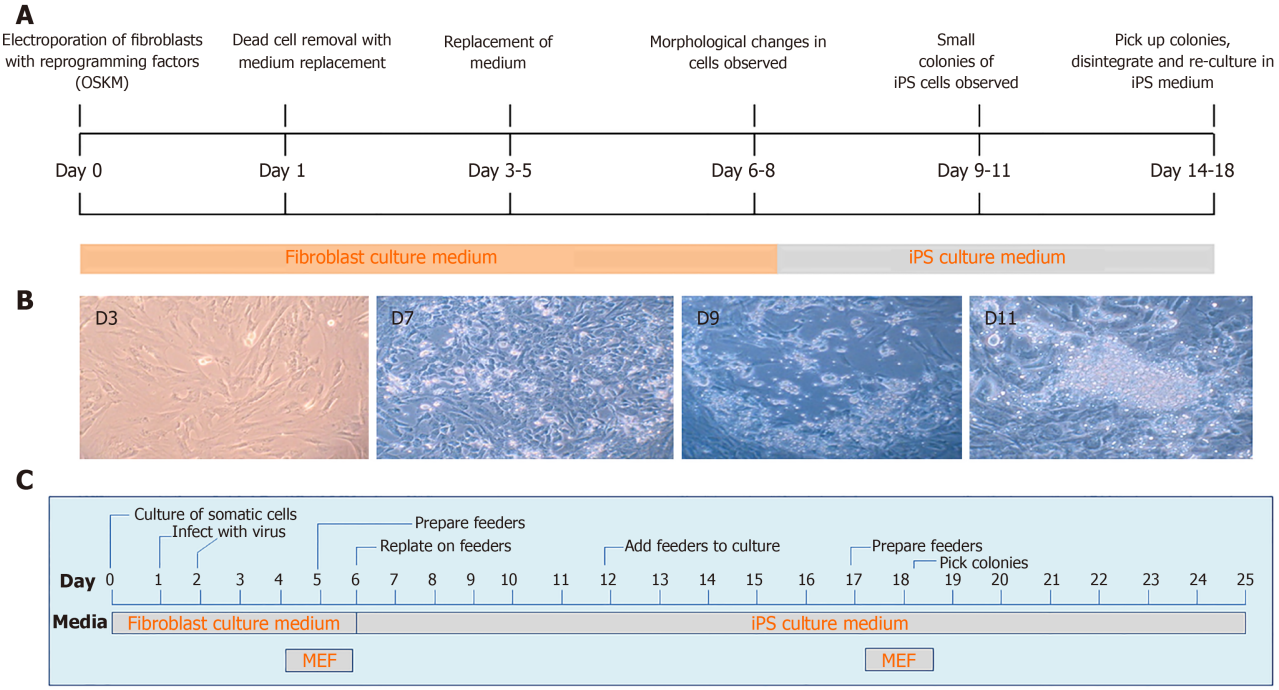

Figure 3 Timeline of transposon-mediated cellular reprogramming of porcine somatic cells to induced pluripotent stem cells (A), change in the morphology of somatic cells in the culture after transposition (B, unpublished own data), timeline of virus mediated cellular reprogramming of somatic cells to induced pluripotent stem cells (C). iPS: Induced pluripotent stem; MEF: Mouse embryonic fibroblast.

our knowledge, there is no report on the successful transposon-derived iPS cell-mediated germline contribution in large domestic animals.

The iPS cells may be directed into the lineage of interest by supplementing various growth factors into the culture media. These growth factors or stimulating agents allow directed differentiation of iPS cells towards a particular cell lineage or cell type. The differentiated cells can be identified with the help of various markers, which are highly expressed in these cells. Very few markers are specific for one cell type, and as such, a panel of markers needs to be used in order to characterize the differentiation status. In this direction, EBs derived from SB-mediated mouse iPS cells were differentiated into cardiac cells with a beat frequency[21,23]. Davis *et al*[20] observed that SB-mediated human iPS cells differentiated into EBs which contained hemoglobinized erythroid cells as well as spontaneously contracting cells, indicating that iPS cells could be differentiated into hematopoietic cell types and cardiomyocytes.

EBs generated from PB-mediated rat iPS cells showed numerous Alcian blue-stained regions, indicating the presence of acidic proteoglycans[29]. These acidic proteoglycans were suggestive of cartilaginous tissue, which was further confirmed by the production of collagen II. Transgene-free human iPS cells derived from PB reprogramming were successfully differentiated into epidermal keratinocytes, which were found to be similar in morphological, functional, and molecular analysis of single-cell gene expression to normal human keratinocytes[116]. The protocol for differentiation of human iPS cells into keratinocytes employed either retinoic acid or bone morphogenetic protein 4 (BMP4)[117]. Igawa *et al*[116] used a modified protocol in which neither BMP4 nor retinoic acid were used. Around 5 weeks of initiation of

differentiation, they reported obtaining keratinocyte-like cells. These cells were propagated through successive passaging at least five times in serum-free keratinocyte medium without feeder cells. Upon characterization, these cells were positive for K5/K14, suggesting successful differentiation of keratinocytes from human iPS cells, and they called these cells induced keratinocytes[116]. These results indicate that iPS cell lines could be selected for therapeutic purposes.

Our group presented a novel approach for the differentiation of murine iPS cells derived through PB-mediated reprogramming into lentoid bodies[118]. We established a co-culture system using human NTERA-2, a committed neuronal precursor cell line[119] and P19, a murine embryonic carcinoma cell line[120] to provide a suitable niche for differentiation of the iPSs into the ectodermal lineage. The developing lentoid bodies were identified by a lens lineage-specific reporter, but also showed changed light refraction in the bright-field view. The existing data support the notion that the specific cell type reporter approach is instrumental for the optimization, development and validation of differentiation protocols for murine iPS cells. We speculate that the gained knowledge can be translated to optimize the differentiation of lens cells from human iPS cells and thus to advance the progress of patient-specific lentoid bodies as a pipeline for *in vitro* drug testing. It is likely that the specific cell type reporter approach is also adaptable for *in vitro* tracking of other cell lineages.

TRANSPOSON-BASED SYSTEMS FOR CELLULAR THERAPY

Cell-based therapy aims to treat diseases which cannot be addressed adequately by existing pharmaceutical interventions. The technology utilizes the cells with the ability to differentiate into specific lineages that are subsequently administered to a patient for therapeutic treatment. For this purpose, stem cells are considered ideal to restore tissue repair, or to replenish cells in the background of a genetic disease. The iPS cells can be expanded indefinitely and they are capable of differentiating in all the derivatives of the three germ layers. The generation of iPS cells is without the ethical stigma associated with ES cells, and iPS cells are able to result in personalized stem cells created from patient-specific cells. Although viral vectors are one of the most used methods for cellular reprogramming, their inherent limitations do not favor their clinical application due to hurdles in large-scale vector production and require careful biosafety characterization, which majorly impacts the costs of clinical-grade production of reprogrammed cells.

In recent years, non-viral DNA transposon based-systems have emerged as a potential tool to overcome some of the above-mentioned limitations. In transposon-mediated genetic manipulation, gene(s) of interest such as therapeutic gene rendering stable phenotypic correction, can be introduced and the resulting stem cells can be expanded *in vitro* and then subjected to differentiation into particular cell lineages according to the therapeutic need. The iPS cells generated through transposon-mediated cellular reprogramming are capable of differentiation into EBs *in vitro* and readily form teratomas *in vivo*. Teratoma formation confirmed that the reprogrammed iPS cells had the developmental potential to produce tissues of all three primary germ layers, *i.e.*, ectoderm, mesoderm and endoderm[23,27,28,30,31]. However, the gold standard of the iPS cells pluripotency is determined by their ability to form germline-competent chimeras. Woltjen *et al*[16] demonstrated the formation of murine chimeras from transposon-reprogrammed iPS cells. However, most of the currently used transposon-mediated iPS cell lines carry constructs driven by a strong promoter, which constitutively promotes the reprogramming factors that will prevent the contribution to a normal ontogenesis[25,26,30]. Thus, the transposon-mediated iPS cell lines in several species have not yet been tested for their capability to generate chimera and mediate germline transmission. The recent progress achieved in the area of integration-deficient, but excision-competent transposase variants[61] will further simplify the transposon removal after complete reprogramming and the achievement of autonomous stemness.

Several advantages of transposon systems have encouraged investigators to carry out a clinical trial for the treatment of B-cell malignancies using SB-modified T-cell therapy[121]. The results published in 2016 showed that the use of SB-modified chimeric antigen receptor (CAR) T-cells is safe when infused after allogeneic or autologous hematopoietic stem cell transplantation as an adjuvant therapy. Modified cells survived for an average of 51 or 201 d in the allogeneic or autologous setting, respectively, and patients showed progression-free survival rates that were improved when compared to historical data[122]. Thereafter, iPS cell-based clinical trials have been

initiated to treat Parkinson's disease, heart disease and macular degeneration, highlighting the rapid progress that continues to be made in this area[123,124]. To treat Duchenne muscular dystrophy, Filareto *et al*[125] showed that SB-mediated ectopic expression of micro-utrophin in dystrophic iPS-derived skeletal muscle progenitors restored the muscle pathology by contributing to dystrophin–glycoprotein complex formation, which resulted in improved muscle contraction strength. PB-mediated expression of drug-inducible *MYOD1* gene in human iPS cells lead to more efficient differentiation into myocytes[102]. Similarly, SB-mediated overexpression of PAX3 in iPS cells induced differentiation into MYOD positive myogenic progenitors and produced multinucleated myofibers[126]. Transposon-mediated iPS cells derived from patients suffering from either sickle cell disease caused by a β-globin gene mutation or Huntington's disease caused by trinucleotide repeat expansions in the Huntingtin gene were successfully used for gene editing[127-129]. The most commonly used transposons PB and SB were successfully used to generate human iPS cells from patient-derived cells with a disease-causing genetic background[16,22,130]. These studies indicated that transposons are capable of introducing functional gene copies in patient-derived iPS cells containing defective genes. Recent evidence showed that transposon-mediated gene transfer was demonstrated in several types of cells such as ES cells, iPS cells, CD34+ hematopoietic stem cells or myoblasts[131].

Transposon-based gene delivery could also be used in combination with designer nucleases in iPS cells to correct gene defects. Yusa[132] reported that the endonuclease-based gene targeting efficiency increased using the PB transposon and it occurred due to the possibility of seamless removal of the drug marker enabled by re-transfection of the transposase. More recently, a transposon system was used in combination with *CRISPR/Cas9* for the generation of iPS cells from Huntington disease patients to correct mutations in the Huntingtin gene and corrected cells were then differentiated successfully into excitable, synaptically active forebrain neurons[129]. Similarly, Wang *et al*[94] demonstrated that PB in combination with CRISPR/Cas9 for genome editing in iPS cells, in which the transposon delivered *Cas9* gene followed by delivery of sgRNA caused modification. Subsequent transient transposase expression of inducible Cas9 cassette was removed and yielded genome-edited iPS cells with seamless transgene removal.

The treatment of several human diseases often involves genetic manipulation of iPS cells prior to transplantation, which may further threaten their genomic stability. Overall, genomic aberrations can affect differentiation capability, identity and tumorigenicity of iPS cells. In the promising era of iPS cell research and therapy, the genomic stability of iPS cells and their safety, efficiency, and specificity remains one of the highest concerns prior to clinical translation[133]. Hence preclinical trials in mice and other animal models are necessary in the future to confirm the *in vivo* therapeutic potential of reprogrammed cells. Challenges for reprogrammed cells are that they not only contain the *in vivo* delivery and dosage, but also their stability and potential off-target effects[4]. These challenges are currently hindering the progress to translate this potentially promising approach to clinical applications, but they appear to be solvable due to rapidly evolving advances in cellular reprogramming.

POTENTIAL RISKS OF TRANSPOSON-MEDIATED CELLULAR REPROGRAMMING AND THEIR SOLUTIONS

The use of SB systems appears to be safe in human cells with respect to off-target effects, as they originate from fish genomes, and the mammalian genome does not contain sufficient transposons to allow them to be cleavage by the transposase[50,73]. Hence, the SB transposon exhibits the least deviation in genome-wide distribution and no apparent bias was observed for either the heterochromatic or euchromatic region and weak correlation with transcriptional status of targeted genes was detected[134]. In addition, the ITRs region have negligible promoter/enhancer activity, and therefore they are unable to initiate transcription of genes that flank the integration site[135]. This system is highly efficient in transfecting even those cell types which are hard to transfect. On the other hand, PB systems have a wide target site that favor integration into genes and near chromatin marks characteristic of active transcription units[73,134,136,137]. These observations indicate that transposons (SB and PB) might be safe for therapeutic gene delivery in clinical trials.

After delivery of the transposon system, the transposition may undergo multiple rounds of remobilization[138,139], which should be minimized by carefully controlling the transposase dose[136]. In mouse embryonic stem cells approximately 95% of genomic

transposon excision was reported to be precise and 5% of the transpositions showed genomic alterations[138]. It was also observed that frequent transposition into unknown sites could result in micro-deletions, footprint mutations as well as chromosomal rearrangements in the genome, which makes it labor intensive to identify integration-free iPS cells with intact genomes[138,140]. As a consequence, the transposase expression window should be tightly controlled to achieve traceless excision without inducing any genomic alterations and cytotoxicity[141].

Due to its non-viral nature and integration capacity, some of the transposon systems were adapted for use in gene therapy practices. To achieve efficient and safe use, the transposon systems were split into two plasmids, one containing the sequence encoding the transposase enzyme and the other comprising an expression cassette flanked by ITRs. However, in spite of these advantages, DNA transposon based vectors are essentially gene-inserting tools that still need assistance for efficient cellular uptake. Therefore, its activity depends on cell type, transfection method, and plasmid size. Moreover, it is important to note that these vectors have been largely used in the preclinical setting, and clinical trials are in progress to evaluate their efficacy, safety and presumed advantages.

Transposon-based gene transfer followed by cellular reprogramming might be associated with the important risk factor of genotoxicity. The genotoxicity could be induced either by interaction of the transposase with endogenous DNA sequences, or the genome-wide insertion profile of the transposon vector. To increase the efficacy and safety of cellular reprogramming, many efforts have been made to obtain potential molecules that can improve reprogramming efficiency or replace some of the vital transcription factors[142]. In this direction, various small molecules such as histone deacetylase inhibitors, DNA methyltransferase inhibitors, methylases, and demethylase inhibitors, Rho-associated protein kinase, and Wnt pathway regulators have been recognized to be effective in inducing reprogramming of terminally differentiated cells[143-146]. Huangfu *et al*[147] showed that valproic acid, a histone deacetylase inhibitor, increased the efficiency of transcription factor-mediated cellular reprogramming from 0.50% to 11.8%, indicating chromatin modification is one of the major rate-determining steps during cellular reprogramming. In addition to these, other molecules have also been tested to improve cellular reprogramming efficiency, including RepSOX2, E-616452 (2-[3-(6-methyl-2-pyridinyl)-1H-pyrazol-4-yl]-1,5-naphthyridine), and OAC1 (Oct4-activating compound 1), which facilitate the mesenchymal-epithelial transition (MET), and activate the stemness-associated promoter regions of mature fibroblasts[148,149]. Nowadays, the use of these small molecules is more trustworthy for introducing transcription factors into cells, but it remains a challenge to break through the efficiency threshold due to inadequate gene delivery and limitations in cellular uptake[150].

As compared to integration of retrovirus[151] and lentivirus[152], the integration profile of PB[137] and SB are safe, and are currently being tested for several clinical trials of T cell immunotherapy. Furthermore, to exclude the possibility of remobilization, the transposase could be transfected in the form of RNA, which seems to be less toxic to the cells[153].

CONCLUSION

Recently, transposon systems have been developed as attractive tools for somatic cell reprogramming, which has significant potential in speeding up patient-specific cell based therapies, as they can overcome some of the limitations of viral-based reprogramming technologies[125,154]. Furthermore, transposon systems have unique features for excising the exogenous reprogramming cassette from the iPS genome through re-expression of the transposase. Transposon systems eventually gives rise to "transgene-free" iPS cells, which is valuable in minimizing the risk of reactivation of reprogramming factors with oncogenic potential[94]. In addition to gene delivery, gene correction can also be achieved with a combination of transposons and designer endonucleases including ZFN, TALEN or CRISPR/Cas9. The introduction of a site-specific DNA double-strand break by endonuclease activity allows homologous recombination at target genes, followed by traceless removal of selectable gene cassettes by the transposase. This strategy has been used in SCD patient-derived iPS cells without any detectable off-target activity and undesirable chromosomal alterations[127]. More recently, the practice of mRNA encoding transposases to prevent continued mobilization of transposons and modification of ITRs, and the generation of hyperactive and codon-optimized transposase variants enhanced the overall

transposition efficiencies[88]. This broadens the spectrum of possible therapeutic alternatives for gene therapy in particular, and gene correction in iPS cells. A number of preclinical studies performed as disease models that simulate the cognate human disorders have highlighted the potential of transposons for gene therapy[154]. Thus, iPS cell biology will continue to play a major role not only in the advancement of medical sciences, but also in improving the understanding of basic sciences. Looking forward, the continued advancement and refinement of transposon based-technologies and the steps toward their clinical translation will likely herald an exciting era in gene therapy.

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REVIEW

Approaches to promoting bone marrow mesenchymal stem cell osteogenesis on orthopedic implant surface

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Abstract

Bone marrow-derived mesenchymal stem cells (BMSCs) play a critical role in the osseointegration of bone and orthopedic implant. However, osseointegration between the Ti-based implants and the surrounding bone tissue must be improved due to titanium's inherent defects. Surface modification stands out as a versatile technique to create instructive biomaterials that can actively direct stem cell fate. Here, we summarize the current approaches to promoting BMSC osteogenesis on the surface of titanium and its alloys. We will highlight the utilization of the unique properties of titanium and its alloys in promoting tissue regeneration, and discuss recent advances in understanding their role in regenerative medicine. We aim to provide a systematic and comprehensive review of approaches to promoting BMSC osteogenesis on the orthopedic implant surface.

Key Words: Bone marrow mesenchymal stem cells; Osseointegration; Orthopedic implant; Biofunctionalization

Core tip: Bone marrow-derived mesenchymal stem cells (BMSCs) play a key role in tissue repair after bone and joint injures. The effects of the surface treatment of the orthopedic implants on the osteogenic differentiation of BMSCs are worthy of attention. In this paper, we review recent advances in approaches that promote osseointegration of BMSCs on the surface of orthopedic implants.

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INTRODUCTION

Since Friedenstein *et al*[1] isolated bone marrow mesenchymal stromal cells from for the first time and regarded them as bone tissue progenitor cells, they have played an increasingly important role in orthopedics. Bone marrow-derived mesenchymal stem cells (BMSCs) are ideal candidates for tissue repair after traumatic injury because they are relatively easy to harvest *in vitro* and can undergo self-renewal and multi-directional differentiation into several mesodermal and non-mesodermal cell lineages including osteoblasts, chondrocytes, and adipocytes[2-6]. Degenerative diseases of bone such as osteoarthritis can lead to bone fractures and immobility, compromising quality of life. In the treatment of osteomyelitis, after effectively controlling the symptoms of infection using local or systemic antibacterial drugs, BMSCs differentiate into osteoblasts and lipoblasts, and finally differentiate into mature bone adipose tissue for repair local injury[7]. However, although much attention has been paid to the engineering of biomaterials that regulate BMSC commitment to specific lineages, like the chondrogenic and osteoblastic lineages, harnessing BMSC fate remains a major challenge[8,9]. Therefore, overcoming these challenges would be very significant in the field of orthopedics, where the ability to stimulate osteogenic BMSC differentiation on biomaterials like titanium and its alloys would translate into higher rates of implant osseointegration and improved long-term functionality. In addition, it is necessary to stimulate the *in vivo* environment using BMSCs to study the cellular response at the bone-implant interface since BMSCs are in direct contact with the implant after surgery[10].

The term "osteointegration" has been used since Professor Branemark first reported the phenomenon of "osteointegration" to describe the stable combination of biomaterials and bone tissue. Osseointegration refers to the direct contact of the bone with the implant without an intermediate layer of connective tissue. This biological fixation is a prerequisite for implantable prostheses and their long-term success.

Titanium and its alloys have been widely used in biomedical areas in recent decades for cardiovascular, orthopedic, and dental applications due to their resistance to fatigue, superior mechanical properties, and load-bearing capabilities[11,12]. For example, the elastic modulus of nitinol is 40 GPa, compared to 30 GPa for bone[13]. However, there are major disadvantages to using Ti-based implants, including inert biomaterials and poor biological activity[14,15]. In addition, they fail to achieve sufficient osseointegration, leading to increased aseptic loosening and premature implant failure[16,17]. Therefore, these problems with Ti-based implant materials have hindered to some extent their development as orthopedic implants. Campoccia *et al*[18] believed that the surface of an ideal osteo-compatible biomaterial should possess the following characteristics *in vitro*: (1) Allow good and tight initial adhesion; (2) Support cell attachment and viability; and (3) Have a positive influence on the osteogenic differentiation process. Given that the interaction between the implant materials and bone tissue first occurs on the implant surface, it is necessary to modify the implant surface to solve the problems in titanium and its alloys. BMSCs have the critical role to achieve bone and implant osseointegration. Surface composition, hydrophilicity, and roughness of the orthopedic implant can affect BMSC differentiation and affect osseointegration. Thus, the surface of the implant must be biomodified to create a bioactive surface that is helpful to promote cell-material interactions and improve osseointegration of titanium and its alloys[19-21].

Many surface modification techniques like physical vapor deposition, sol-gel, ion implantation, anodization, and micro-arc oxidation have been investigated to improve the surface properties of titanium and its alloys[22-25]. Although many researchers pay attention to the effect of titanium surface modification on its biological activity, there are still few studies on the effect of modification of titanium and its alloys on the behavior of mesenchymal stem cells. The main aim of this review study is to report the state of art on the technological advancements of titanium implant surfaces to promote osteogenic differentiation of BMSCs on orthopedic implants. This review article deals with the titanium properties, innovative physicochemical procedures to modify titanium surfaces, biomimetic functionalization, promotion of BMSC osteogenesis, and inhibition of biofilm accumulation. We hope that it can provide some ideas for better methods to improve osseointegration efficiency.

BIOFUNCTIONALIZATION OF ORTHOPEDIC IMPLANT WITH BIOACTIVE CERAMIC TO REGULATE BONE MARROW MESENCHYMAL STEM CELL BEHAVIOR

Bioactive ceramic materials have a certain degree of solubility in the body, releasing certain ions that are not harmful to the body, participating in organic metabolism, stimulating or inducing bone hyperplasia, promoting defective bone tissue repair, and showing other good biological properties. This type of material may contain hydroxyapatite, or it can produce hydroxyapatite after reacting with body fluids, which can be integrated with bone tissue to form a bone binding interface. This method belongs to chemical combination with high strength and good stability. In this section, we will review the effects of different methods of bioactive ceramic coating on the behavior of BMSCs. The overall situation is shown in Table 1.

Plasma spraying

Plasma spraying technology is a method in which a plasma electric arc driven by a direct current is used as a heat source to heat materials like ceramics, alloys, and metals to a molten or semi-fused state before spraying the surface of a pretreated workpiece at a high speed to form a firmly adhered surface layer. Plasma spraying is an effective method to prepare bioactive ceramic coatings. Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HA] is a calcium hydroxide and tricalcium phosphate compound salt with a chemical composition and crystalline structure similar to the main minerals in human bones and teeth. It is also the main inorganic component of human bone tissue, and a typical bioactive material with good biocompatibility and chemical stability. It has been reported that spraying a hydroxyapatite ceramic coating on the surface of titanium-based implants leads to good cellular compatibility, promotes adhesion, proliferation, and osteogenic differentiation of BMSCs, and improves the implant's bond to surrounding bone tissue. In one study, Dimitrievska *et al*[26] fabricated a new type of titanium alloy that possesses a layer of hydroxyapatite on titanium dioxide by plasma spraying. They studied the behavior of BMSCs on this titanium-based material. The results show that cells have stronger initial adhesion (improved by 20% after 2 h) and higher metabolic activity (improved by 20% after 2 h) on TiO_2-HA compared to the titanium dioxide group. Furthermore, the differentiation of BMSCs is evidenced by alkaline phosphatase (ALP) and osteocalcin (OCN), early indicators of osteogenic differentiation, which are significantly increased on TiO_2-HA. However, the pure HA coating also has some serious defects: High brittleness, poor fatigue resistance, and weak bonding strength with metal substrates. Porous tantalum has attracted much attention for its good biocompatibility and microstructure similar to cancellous bone[27]. In a recent study, Ta-incorporated HA coatings were developed by Lu *et al*[28] using the plasma spray technique on a titanium substrate. The result demonstrated that Ta-incorporated HA coating could promote initial adhesion and faster cell proliferation after incubation for 3 and 5 days, but it also promotes osteogenic differentiation of BMSCs compared to HA coatings. Akermanite ceramics can induce apatite mineralization. They also have moderate stability in simulated body fluid (SBF) and generally good mechanical properties, and support BMSC attachment[29,30]. The researchers found that the bonding strength between the plasma-sprayed akermanite bioactive coatings and Ti substrates is higher than hydroxyapatite (HA) coatings, and BMSC attachment and proliferation were more significant on akermanite coatings than on HA coatings[31].

Sol-gel method

Sol–gel process first described 150 years ago is still receiving great attention as one of the easiest ways to develop modified materials which possess required properties and are characterized by durability and stability[32]. Hence, sol-gel process is another method for preparing bioactive ceramic coatings. The sol-gel technology has some advantages compared to plasma spraying methods, including chemical uniformity, fine grain structure, and lower processing temperature[33]. In a study, a micro/nano-layered structure was prepared on a micro-structured titanium (Micro-Ti) substrate using a sol-gel method with a spin coating technique. The results confirmed that the micro/nano-level structure of large particles (80 nm) significantly promoted MSC proliferation and differentiation compared to other small particles (20 nm and 40 nm)[23]. Inzunza *et al*[34] prepared nanoporous silica coatings on Ti using the sol-gel method and evaporation-induced self-assembly method. The silica coatings with highly ordered sub-10 nm porosity accelerate the adhesive response of early BMSCs and promote BMSC osteogenic differentiation.

Table 1 Biofunctionlization of orthopaedic implant with bioactive ceramic to regulate bone marrow mesenchymal stem cell behavior

Method	Preparation of bioactive ceramic	Cell response	Ref.
Plasma spraying	TiO_2-HA nanocomposite powders were thermally sprayed *via* the HVOF (high-velocity oxy-fuel) technique.	HBMSCs have stronger initial adhesion and favor osteogenic differentiation.	Dimitrievska *et al*[26]
	Ta-incorporated HA coatings were fabricated using the plasma spray technique on a titanium substrate.	Ta-incorporated HA coating could promote initial adhesion, faster proliferation, and osteogenic differentiation of BMSCs.	Lu *et al*[28]
	An atmosphere plasma spray system was applied to spray the synthesized 40-80 μm powders onto the treated substrates.	The attachment and proliferation of BMSCs were more significantly on akermanite coatings than on HA coatings.	Yi *et al*[31]
Sol-gel method	Ti disks were etched with the mixed solution of HF and H_2SO_4. Next, EtOH solutions containing tetrabutyl titanate (TBT) were spin-coated onto samples.	The micro/nano-level structure of large particles (80 nm) significantly promoted MSC proliferation and differentiation.	Shen *et al*[23]
	Pre-hydrolyzed silica solution was added to a solution containing the pores structure-directing agents dissolved in ethanol.	The silica coatings accelerate the adhesive response of early BMSCs and promote BMSC osteogenic differentiation.	Inzunza *et al*[34]

HVOF: High-velocity oxy-fuel; HBMSCs: Human bone marrow-derived mesenchymal stem cells; HA: Hydroxyapatite; BMSCs: Bone marrow-derived mesenchymal stem cells; HF: Hydrofluoric acid; H_2SO_4: Sulfuric acid; TBT: Tetrabutyl titanate; MSC: Mesenchymal stem cell.

SURFACE TOPOGRAPHY TO REGULATE BONE MARROW MESENCHYMAL STEM CELL BEHAVIOR

A bioactive ceramic layer is coated on the surface of porous titanium, and its osteoconduction supports the new bone to grow into the pore along the pore wall, which can effectively improve biological fixation of the porous titanium coating. However, this method also has some shortcomings: (1) After applying the bioactive ceramic coating, the pores of bone tissue are reduced, so the contact area with bone tissue is reduced; and (2) Bioactive ceramic coating still has degradation, poor combination with titanium, and other problems[35]. A variety of surface modification methods have been developed to improve titanium bioactivity. In this section, we will review the different surface modification methods to provide a reference for clinical use (Table 2).

Chemical treatments

Chemical methods can be used to increase the thickness of the oxide film to improve the biocompatibility and bioactivity of titanium and its alloys. The surface chemical treatment of titanium and titanium alloys mainly includes alkali treatment, acid treatment, and acid-base two-step treatment. Alkali solution is used to modify the titanium surface to obtain sodium titanate gel with rich Ti-OH groups on the surface, endowing it with biological activity[36,37]. For this purpose, Cai and his team employed potassium hydroxide to modify the surfaces of titanium substrates; the formed potassium titanate layer enhances titanium's corrosion resistance. The proliferation and differentiation levels of alkaline phosphatase and osteocalcin were significantly increased in MSCs cultured on alkaline-treated titanium after 7 and 14 d of culture, respectively[38].

Acid treatment is often used to remove the oxide layer and contaminants on the surface of the medical titanium material to obtain a clean and uniform surface. The acid treatment results in a 10-nm thick oxide layer, while the titanium oxide in the air is only 3-6 nm thick[39]. Maekawa *et al*[40] treated titanium with polyphosphoric acid solution for 24 h at 37 °C. Surface texture measurement results show that the maximum surface roughness of the treated titanium surface significantly increased. Significantly higher cell attachment and proliferation were also found on titanium treated with polyphosphoric acid in contrast to untreated titanium (control). By comparing the effects of acid-treated titanium and pure titanium on osteogenic differentiation of bone MSCs, Perrotti *et al*[41] concluded that 1 wk of treatment was more than enough for osteoblast differentiation on acid-treated titanium. Silva and his group suggested that rough surfaces submitted to acid-etching favor undifferentiated mesenchymal cell differentiation into osteogenic lineage cells compared to smooth titanium surfaces without acid treatment[42]. Although many studies have shown that surface acidification can increase the degree of roughening and improve the biological activity of titanium implants, acid treatment may cause hydrogen to penetrate below

Table 2 Surface topography to regulate bone marrow mesenchymal stem cell behavior

Method	Treatment process	Cell response	Ref.
Chemical treatments	Commercial pure Ti was immersed into KOH solutions.	The differentiation levels of ALP and OCN were significantly increased.	Cai *et al*[38]
	The Ti disks were immersed into solutions of polyphosphoric acid.	Significantly higher cell attachment and proliferation were also found on Ti treated with polyphosphoric acid.	Maekawa *et al*[40]
	Surfaces submitted to polishing plus etching with 0.8% HF, 13% HNO_3 solution.	Rough surfaces submitted to acid-etching favor undifferentiated BMSCs into osteogenic lineage cells.	Silva *et al*[42]
	The Ti disks were pickled in oxalic acid solution and NaOH, respectively.	Although BMSC adhesion and osteogenesis were promoted, proliferation was significantly inhibited on treated surfaces.	Li *et al*[47]
	The titanium was treated with H_2O_2.	H_2O_2-treated surfaces were beneficial for promoting BMSC attachment, proliferation, and osteogenic differentiation.	Daw *et al*[52]
	The anodic oxidation was carried out to prepare nanotube on titanium surface.	NT30 supported adhesion, stretching, proliferation, and osteogenic differentiation of BMSCs.	Xu *et al*[24]
Electrochemical anodization	Nanonets on titanium surfaces were prepared.	BMSC cultured on nanonets structured Ti surfaces present a high frequency of alignment.	Grimalt *et al*[53]
	The Ti disks were micro-arc oxidized in an electrolyte solution.	The MAO-coating significantly promoted adhesion and osteogenic differentiation of BMSCs by mediating the integrin β1 signaling pathway.	Li *et al*[57]
	O-PIII treatment was performed in a high-vacuum chamber with a radio frequency plasma source.	O-PIII treatment could enhance the adhesion of BMSCs.	Yang *et al*[59]
Plasma ion implantation and deposition	O-PIII treatment was performed in a high-vacuum chamber with a radio frequency plasma source.	The group treated with the highest concentration of oxygen ions has the best effect on adhesion, migration, proliferation, and differentiation of BMSCs.	Yang *et al*[60]
	The Ti-based alloy was modified by electropolishing and plasma electrolytic oxidation process.	The calcium-ion-implanted titanium remarkably improved BMSC adhesion and proliferation compared to the untreated sample.	Michalska *et al*[61]
	Highly ionized Ca and Mg plasmas were generated from a filtered vacuum arc source and accelerated within the electric field between a sheath and the substrates.	Initial cell attachment on a titanium surface can be improved by Ca and Mg ion implantation. In addition, the expression of osteogenic-related genes like $RUNX_2$ and type I collagen was higher in the Mg ion-implanted surface.	Won *et al*[62]
	The Ti discs were polished with abrasive grit (grades 240–600), and then treated with laser radiation at various fluences (132, 210, or 235 J/cm^2).	Laser-modified titanium surfaces could enhance upregulation of expression of the osteogenic markers and enhance alkaline phosphatase activity of BMSCs.	Bressel *et al*[66]
Laser beam treatment	DMLS discs were fabricated in an argon atmosphere with Yb fibre laser system.	Topographical cues of DMLS surfaces could enhance BMSC adhesion, as well as osteogenesis.	Zheng *et al*[67]
	The laser system was a Ti: Sa laser chain, which delivers 120 fs, 800 nm pulses at a repetition rate of 5 kHz.	BMSCs exhibited a more elongated, spindle-like morphology and higher spreading speeds on FS laser-modified surfaces.	Dumas *et al*[68]

Ti: Titanium; KOH: Potassium hydroxide; ALP: Alkaline phosphatase; OCN: Osteocalcin; HF: Hydrofluoric acid; HNO_3: Nitric acid; BMSC: Bone marrow-derived mesenchymal stem cell; NaOH: Sodium hydroxide; H_2O_2: Hydrogen peroxide; MAO: Micro-arc oxidation; O-PIII: Oxygen plasma immersion ion implantation; Ca: Calcium; Mg: Magnesium; $RUNX_2$: Runt-related transcription factor 2; DMLS: Direct metal laser sintering: Yb: Ytterbium; FS: Femtosecond.

the oxide layer, thereby triggering hydrogen embrittlement[43].

The acid-alkali two-step method is also used for titanium surface modification. Strong acid erosion could cause micropores on the surface of titanium and titanium

alloys to increase surface area. Meanwhile, alkaline solution can form a thicker microporous titanium oxide layer on the titanium surface, improving the titanium implant's biological activity[44-46]. Li *et al* [47] first placed titanium in oxalic acid solution (5 wt%) at 100 °C for 2 h to remove the oxide layer and acquire a homogeneous micropit surface. Each pretreated titanium plate was treated in 5 mmol/L NaOH solution at 80 °C for 24 h. An *in vitro* cell experiment demonstrated that BMSC adhesion and osteogenesis can be better promoted on a micro/nanoporous surface than on an acid etched titanium surface. However, BMSC proliferation was significantly inhibited on treated surfaces after culturing for 4 and 7 days, which may due to the high pH around the implant. The high pH at the cell/material interface may cause alkalosis and inhibit BMSC proliferation and viability[48].

Hydrogen peroxide can also be used for activation treatment of titanium. Hydrogen peroxide treatment of titanium is a chemical dissolution and oxidation process, which could alter surface roughness, thickness, and hydrophilicity, with improvements in titanium osteoconductivity[49-51]. In one study, titanium was treated with 30% volume (v/v) of H_2O_2 (5 mL H_2O_2/g disc) for different times in an unsealed covered container under darkness at room temperature. The modifications induced by 6-24 h H_2O_2-treated surfaces are most beneficial for maintaining or promoting the attachment, proliferation, and osteogenic differentiation of BMSCs[52].

Electrochemical anodization

Anodization refers to the use of an electric field and various dilute acids as electrolyte solutions. A series of REDOX chemical reactions take place on the anode surface to form an oxidation layer. Due to anodization's simplicity, versatility, and low cost, it has gained widespread attention in the surface treatment of titanium implants. In a study, Xu *et al*[24] found that tube diameter had a significant effect on adhesion, proliferation, and differentiation of MSCs. Titanium was used as the working electrode, platinum sheet was used as the cathode, and 0.50 wt% NH_4F + 10 vol% H_2O mixture was used as the electrolyte. The anodic oxidation was carried out at 10, 30, and 60 V, which were designated as NT10, NT30, and NT60, respectively. Finally, NT10, NT30, and NT60 were obtained with pore diameters of 30, 100, and 200 nm, respectively. By comparison, although NT60 can promote osteogenic differentiation to the greatest extent, it significantly inhibits cell adhesion and proliferation. NT10 can promote cell proliferation and adhesion, but it is useless for osteogenic differentiation of cells. NT30 supported adhesion and proliferation of BMSCs, and the cells on NT30 became increasingly elongated with increased diameter and showed a large number of prominent filamentous pseudopods. Moreover, it showed better osteogenesis-inducing ability. In another study, Grimalt *et al*[53] produced a nanonets structure on titanium discs. BMSCs cultured on nanonet structured titanium surfaces present a high frequency of alignment and promote osteogenic differentiation of the cells, while cells on untreated titanium surfaces exhibited a random orientation.

Micro-arc oxidation (MAO) is a new type of anodic oxidation technology that deposits a ceramic coating on the metal surface, and it has been widely applied in the surface modification of titanium and its alloys to enhance biological activity and osteogenic capacity. Based on ordinary anodization, arc discharge is used to enhance and activate the reaction occurring on the anode, thereby forming a ceramic film *in situ* on the surface of titanium[54-56]. Zhou *et al*[25] reported that porous coatings prepared by MAO promote BMSC adhesion and osteogenic differentiation. In addition, the larger the pore size, the more conducive to BMSC adhesion and osteogenic differentiation when the pore size is in the range of 3-10 μm. A similar phenomenon was observed in BMSCs in another study. Li *et al*[57] developed two kinds of coatings (MAO and MAO-Alkali coatings) with similar micro-morphologies, both of which significantly promote BMSC adhesion and osteogenic differentiation by mediating the integrin β1 signaling pathway.

Plasma ion implantation and deposition

Plasma ion implantation (PIII) is known to modify the surface and near surface regions of materials, and it has many advantages for surface modification of materials, including the following: (1) Changing the surface characteristics of the material alone without affecting the properties of the material; (2) The modified layer will not fall off or fail in combination; and (3) PIII is a low-temperature process (approximately 100 °C), and there is no change in the size of the workpiece due to thermal distortion.

PIII surface modification mainly uses plasma generated after Ar, N_2, O_2, and other gases or metal gasification to treat the material surface. Under the action of plasma, the surface of the material is bombarded with high-energy particles in the plasma. Chemical bond breakage occurs, and large molecular radicals are generated. At the

same time, the material is etched to change the surface properties. PIII of metal materials can effectively improve the mechanical properties, wear resistance, and corrosion resistance of orthopedic implants, thus enhancing their biocompatibility[58]. Yang *et al*[59] explored the effect of titanium treated with oxygen plasma immersion ion implantation (O-PIII) on the behavior of BMSCs with different oxygen doses. The results showed that O-PIII treatment could enhance BMSC adhesion, and there was no significant difference in the titanium surface treated with O-PIII when the oxygen ion dose differed. In their later study, Yang *et al*[60] compared the effects of three doses of oxygen ion implantation into titanium on BMSC behavior. Among these treated titanium disks, the group treated with the highest concentration of oxygen ions has the best effect on cell adhesion, migration, proliferation, mineralization, and differentiation of BMSCs. It has been reported that calcium-ion-implanted titanium also remarkably improved BMSC adhesion and proliferation compared to the untreated sample[61]. Similarly, other studies have evaluated the response of BMSCs to titanium surfaces that had been implanted with Ca and Mg ions using the PIIID technique. The results showed that initial cell attachment on a titanium surface can be improved by Ca and Mg ion implantation. Cells on the Mg ion-implanted surface showed more extended filopodia after 4 and 24 h of cultivation. In addition, the expression of genes associated with osteogenic differentiation like $RUNX_2$ and type I collagen was higher in the Mg ion-implanted surface[62]. These results are consistent with previous studies showing that significant cytotoxicity was not observed after Mg ion implantation into a titanium implant, and initial BMSC adhesion was improved with resulting osteoblast differentiation enhancement[63].

Laser beam treatment

Laser beam treatment is a controllable and flexible approach to modifying surfaces, which results in surfaces with increased surface area and enhanced wettability, and it displays negligible corrosion and high removal torques of established implants in preclinical bone models[64,65]. Laser-modified titanium surfaces could enhance upregulation of expression of the osteogenic markers and enhance alkaline phosphatase activity of BMSCs[66]. A recent investigation on the direct metal laser sintering (DMLS) titanium surface found that topographical cues of DMLS surfaces could enhance both protein adsorption ability and BMSC adhesion performance. Moreover, DMLS titanium surface could efficiently induce osteogenesis-associated gene expression in BMSCs *via* H3K27 demethylation and increases in H3K4me3 levels at gene promoters after osteogenic differentiation[67]. In another study, dynamic analyses of early cellular events showed that BMSCs exhibited a more elongated, spindle-like morphology and higher spreading speeds on femtosecond laser-modified surfaces compared to commercially pure titanium[68].

COVALENT IMMOBILIZATION BIOACTIVE MOLECULES TO PROMOTE BONE MARROW MESENCHYMAL STEM CELL ADHESION, PROLIFERATION, AND OSTEOGENIC DIFFERENTIATION

The basic principle of the above physical and chemical methods is to change the physical and chemical characteristics of the metal matrix surface to improve the biocompatibility of the material and BMSC growth inductivity, which is an indirect surface modification method. However, the application of biochemical technology proposed by David A Puleo to improve the surface activity of implants provides a different approach to surface modification from the traditional physical and chemical methods[69]. Contrary to topography-based approaches, biochemical surface modification utilizes macromolecules like extracellular matrix components, peptides, cell growth factors, and others to be fixed on the surface of biomaterials to act as receptors for adjacent cells, matrices, and soluble factors, which form a transition layer suitable for living organisms to control the tissue-implant interface[70]. In this section, we list different types of titanium-implant-bound macromolecules that have been shown to influence BMSC behavior. The overall data are listed in Table 3.

Extracellular matrix components

The extracellular matrix (ECM) is composed of several molecules secreted by cells. In addition to providing structural and mechanical support for tissues to interact with cells, these molecules can also bind to soluble molecules like growth factors that are present in extracellular fluid and regulate the occurrence of tissues and physiological

Table 3 Covalent immobilization bioactive molecules to promote bone marrow mesenchymal stem cell adhesion, proliferation, and osteogenic differentiation

Bioactive molecules	Treatment process	Cell response	Ref.
Type I collagen	Titanium fiber meshes were treated with NaOH, followed by p-nitrophenyl chloroformate, and coated with collagen type I.	The modification of titanium fiber meshes can promote BMSC osteogenic differentiation.	van den Dolder *et al*[72]
	Covalent immobilization of collagen on titanium.	Greater regulation effect on BMSC osteogenesis compared to adsorptive immobilization.	Ao *et al*[74]
	Hyaluronic acid was immobilized on titanium surface by layer-by-layer technique.	BMSCs had more lamellipodia and adhered more closely to the covalently immobilized HyA surface.	Ao *et al*[78]
HyA	Covalent immobilization of RGD peptide on titanium surface.	RGD-functionalized titanium can improve early bone growth and matrix mineralization.	Elmengaard *et al*[87], Karaman *et al*[88]
RGD peptide	HBII-RGD was immobilized on the Ti surface.	HBII-RGD-functionalized Ti surfaces could stimulate BMSC differentiation and mineralization.	Guillem-Marti *et al*[90]
Growth factors	Covalently graft EGF and BMP-2 onto the oxide surfaces.	BMSC adhesion and proliferation were dramatically increased by covalently grafting EGF, but covalently grafted BMP-2 did not.	Bauer *et al*[92]
	PDGF-BB loading on titanium nanotube.	PDGF-BB functionalized surfaces significantly enhanced BMSC attachment and osteogenesis-related functions	Ma *et al*[98]

NaOH: Sodium hydroxide; BMSC: Bone marrow-derived mesenchymal stem cell; HyA: Hyaluronic acid; RGD: Arg-Gly-Asp; HBII-RGD: Heparin binding II-Arg-Gly-Asp; Ti: Titanium; EGF: Epidermal growth factor; BMP-2: Bone morphogenetic protein-2; PDGF-BB: Platelet-derived growth factor BB.

activities of cells. The ECM provides a framework for tissue construction and plays an important role in regulating the survival, migration, proliferation, morphology, and other functions of cells in contact with it. Therefore, ECM components are the first choice for the biochemical surface modification of titanium-based bone implant materials.

TYPE I COLLAGEN

Collagen type I, one of the main organic components of bone ECM, is known to play an important role during adhesion, proliferation, and mineralization processes and osteogenic differentiation of cells, and it is an intriguing candidate for surface immobilization[71]. Dolder *et al*[72] showed that the modification of titanium alloy by type I collagen can promote BMSC osteogenic differentiation.

Morra *et al*[73] fixed collagen I to the surface of titanium (denoted as Col-Ti), finding that enhanced BMSC adhesion and cell density on Col-Ti, together with increased cell spreading areas on the microscopic surface morphology. RT-PCR analysis of several osteogenic related genes showed that the titanium surface immobilized on type I collagen could significantly promote BMSC osteogenic differentiation.

In another study, Ao and his team also found that immobilizing type I collagen on a titanium coating could enhance interactions between cells and materials and improve BMSC functions like adhesion, proliferation, and osteogenic differentiation. Furthermore, they compared the effects of different type I collagen fixation methods on BMSC behavior. They concluded that covalent immobilized collagen on titanium coating has a greater regulation effect on BMSC osteogenesis in contrast to adsorptive immobilization, which can be explained from the perspective of increasing the amount of covalently connected collagen and improving stability[74].

HYALURONIC ACID

Hyaluronic acid (HyA) is rich in carboxyl groups, and it is another major ECM component that possesses good biocompatibility, degradability, and low antigenicity, In addition, HyA could enhance cell migration and proliferation[75-77]. Based on HyA's excellent properties, Ao *et al*[78] fabricated a titanium coating modified with HyA by covalent immobilization. They confirmed that BMSCs had more lamellipodia and

adhered more closely to the covalent immobilized HyA surface than untreated samples. Other *in vitro* cell experiments have also shown that HyA immobilization on titanium coatings could significantly enhance BMSC attachment, proliferation, and differentiation. Furthermore, Ao *et al*[79] prepared a stable collagen/HyA (Col/HyA) polyelectrolyte multilayer (PEM) film on a titanium coating using a combination of the layer-by-layer self-assembly technique and covalent immobilization. The results showed that BMSCs displayed a polygonal and fusiform-shaped morphology, and cell adhesion and proliferation on the material were also improved. In other words, the construction of Col/HyA PEMs on TCs improved the cell–material interaction. The induction of osteogenic differentiation was further determined using qPCR, and the results confirmed that stable Col/HyA PEM could significantly enhance BMSC osteogenic differentiation.

Peptide sequence

It has been found that some short peptides in ECM proteins play important roles in cell behavior regulation[80,81]. Among different ECM proteins, fibronectin (FN), a multifunctional cell adhesive glycoprotein, is one of the most well-known and commonly used to functionalize biological materials. It contains several domains that mediate many cellular processes like cell adhesion, migration, growth, and differentiation. The use of FN-functionalized titanium implants has been shown to improve bone conduction capacity for its ability to attach cells to ECM components *via* integrin receptor interactions[82]. Chen *et al*[83] fixed FN on the surface of titanium, and BMSCs exhibited substantial actin polymerization, in the form of lamellipodia, pseudopodia, and actin stress fiber. However, the cells retained a rounded morphology on untreated surface. Besides, FN-functionalized titanium had a significant positive effect on BMSC proliferation compared to the control. However, its use for clinical applications is hampered due to poor stability, high production costs, and poor ECM protein immunogenicity, which have reduced their biomedical potential[84]. The use of ECM-derived synthetic peptides containing the functional domains of ECM proteins is an effective method to overcome these problems. Therefore, the synthesis of short peptide fragments representing ECM proteins and the modification of titanium-based implants have been gradually developed[85,86]. The most commonly used peptide sequence for surface modification is the arginine-glycine-aspartic acid (RGD) motif. RGD-functionalized titanium can improve early bone growth and matrix mineralization, and it can enhance the combination of materials and new bone[87]. There have been several reports on the effects of RGD on BMSCs. In a study, Karaman *et al*[88] covalently attached RGD peptide to titanium discs. The results indicated that RGD peptide treatment significantly enhanced BMSC adhesion and proliferation. Furthermore, this effect was enhanced by combining cold temperature plasma treatment and RGD peptide coating. Consistent with this, Herranz *et al*[89] concluded that the RGD motif was more favorable for BMSC adhesion, proliferation, and osteogenic differentiation in contrast to fibronectin. In another study, Jordi and his group covalently attached a novel molecule on the titanium surface. The novel molecule possesses adhesion capacity by an RGD gain-of function DNA mutation installed to the heparin binding II (HBII) fragment. The presence of RGD in the HBII domain stimulated focal adhesion formation at BMSC edges where filopodia were spikier compared to bare titanium samples with completely round cells. In addition, HBII-RGD-functionalized titanium surfaces could also stimulate BMSC differentiation and mineralization[90].

Growth factors

Growth factors are a class of proteins secreted by cells that act as signaling mediators for the relevant target cells to perform specific behaviors. Growth factors can promote cell proliferation, differentiation, protein synthesis, and migration of specific cells. Growth factors released from the implant surface can increase osteoblast activity and facilitate bone tissue regeneration[91]. Many researchers have been depositing growth factors on biomaterials to affect cell behavior. In one study, Bauer *et al*[92] showed the covalent immobilization of two growth factors, epidermal growth factor (EGF) and bone morphogenetic protein-2 (BMP-2), on the surface of TiO_2 nanotubes and their effects on BMSC behavior. Cell adhesion and proliferation were dramatically increased by covalently grafting EGF on a surface of a 100 nm nanotube, but covalently grafted BMP-2 did not. The result was consistent with the finding of previous studies that BMP-2 promotes BMSC differentiation into osteoblast lineages but does not contribute to the cell attachment, adhesion, or proliferation like EGF[93]. Studies on BMP-2's effect on BMSC differentiation have shown that BMP-2 has a significant effect on osteoblast differentiation potential[94].

Platelet derived growth factor (PDGF) has been shown to play critical roles in bone regeneration after injury, and it significantly contributes to all stages of bone regeneration after trauma[95,96]. Among three types of dimerism, PDGF-AA, -BB, and –AB, PDGF-BB exerts the most potent chemotactic effects on BMSCs[97]. Ma *et al*[98] fabricated a nano-micro hierarchical TiO_2 clustered nanotubular structure using anodization, and PDGF-BB was functionalized with PhoA (11-hydroxyphosphonic acid)/CDI (carbonyldiimidazole). The resulting new material had almost no cytotoxicity to host cells, and it significantly enhanced BMSC attachment and osteogenesis-related functions (early proliferation, extracellular matrix synthesis, and mineralization).

LOCAL CONTROL RELEASE OF BIOACTIVE MOLECULES TO PROMOTE BONE MARROW MESENCHYMAL STEM CELL ADHESION, PROLIFERATION, AND OSTEOGENIC DIFFERENTIATION

Recently, many researchers have focused on biomolecule-controlled release. This controlled release system overcomes the limitation of rapid degeneration and diffusion of biomolecules in the body, which may decrease biomolecule doses, reduce costs, and more importantly, minimize side effects of high-dose biomolecules. An effective controlled-release system can encapsulate bioactive cues in biocompatible and biodegradable microparticles. As the microparticles gradually degrade, biological molecules are released with predesigned dose kinetics over time[99-101]. The key to making bioactive molecules work is their release so that they can induce the required biological response. Many bioactive molecules can be used in this kind of sustained-release system, including growth factors, short peptides, clinical drugs, and others. By sustained release on the implant surface, cell adhesion, proliferation, differentiation, and other behaviors can be regulated, thus improving the implant's biocompatibility. Table 4 lists the commonly used bioactive molecules and their cellular responses reported in the recent literature.

Coating biodegradable polymers is an effective method to control the drug release kinetics from titanium. In a study, Kim *et al*[102] prepared a new dopamine coating that enhances the initial cell adhesion, mitochondrial activity, and proliferation of BMSCs on the titanium surface. Son *et al*[103] successfully developed hydroxyapatite (HA)-titanium disc surfaces immobilized with dexamethasone (DEX)-loaded poly(lactic-co-glycolic acid) (PLGA) particles using a low-temperature high-speed collision method. The evaluation of HA-titanium surfaces with a particle carrier system potently induced BMSC differentiation *in vitro*. This showed that the gene expression levels of *ALP*, *OPN*, *BSP*, and *OC* were enhanced, and these functional surfaces showed greater osteoinductivity than pure-Ti and HA-Ti surfaces. Cheng *et al*[104] used catechol as a template to modify a photo-crosslinked gel-based hydrogel to enhance its adhesion to the titanium surface, thereby improving the coating's stability. Synthetic silicate nanoparticles (SNs) were introduced into the hydrogel formulation. The results showed that the addition of SNs to the hydrogel formulation can promote bone formation when co-cultured with BMSCs, suggesting the potential to promote new bone formation in surrounding tissues.

APPROACH TO INDIRECTLY AFFECT BONE MARROW MESENCHYMAL STEM CELL ADHESION, PROLIFERATION, AND OSTEOGENIC DIFFERENTIATION

In fact, once implanted, metallic implants would adsorb various proteins, elicit a clotting reaction, trigger an innate inflammatory response, and induce the bone regeneration process[105-107]. Intrinsic inflammation is undesirable but inevitable, and the result of the inflammatory response plays a vital role in the formation of new bone in and material around after implantation[108]. Therefore, it is important to take into account the immunomodulatory effects of biological materials[109]. Specifically, macrophages are involved in almost all-natural wound healing processes. Macrophage polarization has an important effect on wound healing and the biological properties of biological materials[110]. As the key participants of innate host immunity, classically (M1) and alternatively (M2) activated macrophages, the two main phenotypes, are pro-inflammatory and anti-inflammatory, respectively[111]. M1 macrophages express

Table 4 Local control release of bioactive molecules to promote bone marrow mesenchymal stem cell adhesion, proliferation, and osteogenic differentiation

Bioactive molecule	Cell response	Ref.
L-DOPA	The new L-DOPA coating enhances the initial cell adhesion, mitochondrial activity, and proliferation of BMSCs on the titanium surface.	Kim *et al*[102]
DEX	The HA-Ti surfaces with DEX carrier system potently induce BMSC osteogenic differentiation *in vitro*.	Son *et al*[103]
SNs	The addition of SNs to the hydrogel formulation can promote bone formation when co-cultured with BMSCs.	Cheng *et al*[104]

L-DOPA: L-3,4-dihydroxyphenylalanine; BMSCs: Bone marrow-derived mesenchymal stem cells; HA: Hydroxyapatite; Ti: Titanium; DEX: Dexamethasone; SNs: Silicate nanoparticles.

high levels of interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1, inducible nitric oxide synthase, and others. M2 macrophages synthesize IL-10, arginase-1, vascular endothelial growth factor A, and platelet-derived growth factor-BB (PDGF-BB), which support the homing, proliferation, and osteogenic differentiation of BMSCs[112].

Successful biomaterial implantation can be achieved by controlling immune system activation. Hence, many researchers have focused on indirectly regulating the behavior of BMSCs by regulating macrophage polarization. In a study, patterned titanium coatings were prepared by combining grit-blasting, ultrasonic washing, and atmosphere plasma spray which copper meshes were applied to block the molten titanium droplet when spraying. Macrophages tend to polarize to M2 on a patterned titanium surface, while macrophages on traditional titanium coatings exhibit higher M1 polarization.

Up-regulation of osteoinductive cytokines was detected, suggesting that macrophages provide a favorable osteogenic microenvironment[113]. In our previous study, a multi-biofunctional titanium implant was fabricated by covalently immobilizing titanium with the bacitracin. *In vitro* cell biology experiments showed that bacitracin-immobilized titanium could inhibit the secretion of inflammatory factors like TNF-α, IL-6, IL-8, and others, which represent M1 polarization of macrophages, and significantly promote the adhesion, proliferation, and osteogenic differentiation of BMSCs[114]. In another study, Ma *et al*[115] evaluated the osteogenic behavior of BMSCs on TiO_2 nanotubular (NT) surfaces in conditioned medium (CM) generated by macrophages. BMSC morphology in CM from macrophages cultured on the NT surfaces was aligned in a consistent direction, while an unordered distribution was observed on the pure titanium surface. In addition, the modified titanium dioxide surface and CM in monocytes cultured on the surface jointly promoted the proliferation, migration, and osteogenic differentiation of BMSCs. The transition of macrophages from M1 to M2 at specific time points is very important for wound healing and tissue regeneration. In a recent study, a dual system hydrogel layer (chitosan/β-glycerophosphate disodium and carboxymethyl chitosan/genipin) of titanium dioxide nanotubes was fabricated to regulate the release of IL-4 and interferon-γ (IFN-γ). In the culture with BMSCs and macrophages, the system showed good cell compatibility and significantly promoted cell proliferation[116].

PERSPECTIVE OF OSTEOGENESIS ON TITANIUM SURFACE

BMSCs are used as core cells for the renewal and repair of local bone, cartilage, and medullary adipose tissue[117]. BMSCs perceive the titanium surface and become activated during the osteogenesis and osteointegration phases. BMSCs then establish contact with the titanium surface and maintain this relationship until they differentiate into osteoblasts and osteocytes, subsequently embedding in the mineralized matrix[118]. At present, many researchers are mainly focused on the effect of different modification methods on the behavior of BMSCs and have made great progress. However, problems also exist in the modified implants such as poor biological safety and poor stability[119].

In addition, it is important to note that there are great limitations to the existing methods of judging osteogenesis on titanium surface, and the current means of skeletal muscles mainly rely on magnetic resonance imaging (MRI), X-ray computed

tomography, and X-rays[120,121]. Nevertheless, there is still no effective method for the bone integration evaluation on metal implants, which can only rely on pathological biopsy examination. Therefore, the evaluation of osteogenesis on the surface of titanium and its alloys *in vivo* may be an important research target in the future. And more in depth basic and clinical research is necessary to develop more products.

CONCLUSION

In this article, we have summarized recent advances in the approaches for surface modification of titanium and its alloys, and systematically elaborated these modification methods and their effects on cell behavior. The methods like sol-gel, ion implantation, anodization, and micro-arc oxidation can promote osteogenic differentiation of BMSCs and improve the rate of osseointegration by changing surface roughness and hydrophilicity, or regulate the microenvironment of the bone-implant interface. We recommend that the application of modern surfaces in the clinical practice of orthopedics be encouraged to increase and accelerate the osseointegration of the implant and its alloys. To the best of our knowledge, few researchers have done similar work, so we hope that our work might develop some ideas for better methods to improve osseointegration efficiency.

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REVIEW

Photodynamic therapy regulates fate of cancer stem cells through reactive oxygen species

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Abstract

Photodynamic therapy (PDT) is an effective and promising cancer treatment. PDT directly generates reactive oxygen species (ROS) through photochemical reactions. This oxygen-dependent exogenous ROS has anti-cancer stem cell (CSC) effect. In addition, PDT may also increase ROS production by altering metabolism, endoplasmic reticulum stress, or potential of mitochondrial membrane. It is known that the half-life of ROS in PDT is short, with high reactivity and limited diffusion distance. Therefore, the main targeting position of PDT is often the subcellular localization of photosensitizers, which is helpful for us to explain how PDT affects CSC characteristics, including differentiation, self-renewal, apoptosis, autophagy, and immunogenicity. Broadly speaking, excess ROS will damage the redox system and cause oxidative damage to molecules such as DNA, change mitochondrial permeability, activate unfolded protein response, autophagy, and CSC resting state. Therefore, understanding the molecular mechanism by which ROS affect CSCs is beneficial to improve the efficiency of PDT and prevent tumor recurrence and metastasis. In this article, we review the effects of two types of photochemical reactions on PDT, the metabolic processes, and the biological effects of ROS in different subcellular locations on CSCs.

Key Words: Cancer stem cells; Photodynamic therapy; Reactive oxygen species; Photosensitizer; Mitochondrial; Endoplasmic reticulum

Core tip: Photodynamic therapy (PDT) is an effective and promising cancer treatment. PDT directly produces reactive oxygen species (ROS) through photochemical reactions. In

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this article, we review the production process of oxygen-dependent exogenous ROS and the possible endogenous ROS generation process after PDT-mediated subcellular organelle stress. The intracellular metabolism of several ROS produced by PDT is analyzed. Given the extremely short half-life and limited diffusion distance of ROS, we explain from the subcellular localization of photosensitizers how PDT affects the characteristics of cancer stem cells through changes in mitochondrial permeability, activation of unfolded protein responses, autophagy and so on.

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INTRODUCTION

Photodynamic therapy (PDT) is an effective and promising cancer treatment. By injecting a tumor-targeted photosensitizer (PS) into the patient's body and directly irradiating the tissue with a laser, a significant tumor ablation effect can be achieved[1]. The space- and time-selective uptake characteristics of the PS protect normal cells, while the laser radiation is directly pointed to the tumor[2]. Thus, PDT is a multiple-targeting method. Since the US Food and Drug Administration listed PDT as a new treatment method for the clinical treatment of cancer patients[3], alone or combined with surgery[4] and/or chemotherapy[5], PDT has been applied in large numbers worldwide. For PDT treatment, some Western countries have established relatively systematic treatment plans[6-8]. The main mechanism of PDT depends on the reactive oxygen species (ROS) components generated by the photochemical reaction, which can oxidize a large number of intracellular active components (such as DNA and lipid compounds) in tumor cells[9,10]. This chemical-dependent treatment is more sensitive than drug treatments and can minimize tumors in the short term.

It is currently recognized that ROS play decisive roles in the biological effects mediated by PDT because PDT is based on a natural cold photochemical reaction[11]. Although PDT can be divided into two types of reactions, depending on the type of PS, the products can both be considered ROS. Essentially, the exogenous ROS induced by this cold photochemical reaction are the most important and first effector molecules of PDT, although they also depend on the intracellular oxygen levels most of the time. In addition to exogenous ROS, PDT-induced ROS can also cause intracellular metabolic changes, induce endoplasmic reticulum stress[12], and/or destroy mitochondrial potential[13] to increase endogenous ROS production. On the one hand, the photochemical reaction directly caused by PDT produces a short ROS duration (< 0.05 μs), with high reactivity and a limited diffusion distance (< 0.02 μm)[14]. Therefore, the main target position of the photochemical reaction in the cell is often near the subcellular components where the PS is localized, which explains the heterogeneity of the effects of different PSs[14]. On the other hand, the metabolic induction of mitochondrial ROS production is much more complicated; for example, it may involve the partial inactivation of respiratory complexes I, II, and III of the mitochondrial electron transport chain[15]. In general, excessive ROS destroy the redox system in cells and cause oxidative damage to biomolecules, including DNA and other molecules[10,11]. In previous studies, DNA was considered an important target of PDT because double-strand DNA breaks are the most lethal form of damage to tumor cells[16]. Recently, more studies have suggested that the activation of the mitochondrial permeability transition increases the levels of reactive nitrogen substances, such as nitric oxide[17,18]. Of course, ROS-induced intracellular metabolism affects mitochondria to an even greater extent. The ROS-related effects on the endoplasmic reticulum, nucleus, and cell membrane are described in detail in a subsequent section.

There is growing evidence that ROS play roles in cell signaling. These signals are transmitted in tissues to coordinate various cellular processes. At physiological doses, ROS maintain cell nutrition and cytokine balance; however, in some specific cases, small changes in ROS level may have a profound impact on the fate of stem cells[19], directly induce cancer stem cell (CSC) differentiation, or induce CSC heterogeneity in tumors[20]. Furthermore, ROS are related to the level of many biological processes, including but not limited to gene expression, protein translation, and protein or

nucleic acid interactions[21]. As genomics and proteomics advance, increasing pathway information on ROS, explaining the mechanisms by which they maintain and regulate cellular processes, is being mined. Especially in stem cells, changes in the oxidative state (also known as redox regulation) may indicate the regulatory elements for communication between key organelles such as endoplasmic reticulum-mitochondria and mitochondria-nucleus crosstalk[22,23]. Redox-mediated mitochondrial-nuclear crosstalk can explain the coordination of cell metabolism and chromatin remodeling, gene expression, cell cycle progression, DNA repair, and cell differentiation. The endoplasmic reticulum-mitochondria crosstalk (as well as that of other organelles with mitochondria) can explain endoplasmic reticulum stress, mitochondrial autophagy, stemness induction, apoptosis, and/or survival through ROS.

ROS are also related to immunogens and tolerogenic processes[24]. The increased systemic immunity or enhanced tumor immunity induced by PDT may also be mediated by ROS[25]. Currently, although little is known about whether or how ROS are involved in stem cell immunity, it has been determined that identifying the mechanism by which ROS metabolism affects the fate of stem cells will promote the necessary understanding to apply PDT to inhibit the spread of distant cancer stem cells. Although PDT is used to treat superficial malignancies, its immunogenicity has the potential to eliminate systemic CSCs. But in a counterintuitive outcome, some research found that low-dose PDT promotes tumor cells metastasis[25,26]. The epithelial-mesenchymal transition (EMT) has been shown to be one of the causes of cancer cell migration and invasion[27]. PDT can induce EMT *in vitro*[28]. EMT may be closely related to the metabolic reprogramming of CSCs and cancer cells[28]. ROS can induce stemness and metabolic changes in cancer cells. Therefore, PDT appears to induce the EMT and promote CSC phenotype acquisition by regulating cellular metabolism. The EMT, stemness, and oncogenic metabolism are known to be associated with resistance to PDT. Therefore, understanding PDT-induced metabolism and the molecular mechanism of the EMT is also conducive to accurately generating the appropriate level of ROS and enhance the efficacy of PDT. Therefore, in this review, we present the differences in ROS produced by the two types of photochemical reactions induced by PDT, the metabolic processes of endogenous ROS, and the similarities and differences in the biological effects of different ROS. We analyze the effects of ROS on cells at different sites and explain how they might affect the fate of stem cells. Finally, in view of some controversial characteristics of CSCs, we propose how to leverage the advantages of PDT to manipulate the fate of CSCs.

PRODUCTION AND METABOLISM OF ROS DURING PDT

Generation of exogenous ROS

PDT is a chemical reaction between a PS and oxygen under laser energy; when the three are combined, ROS are produced. Under normal circumstances, the PS is in the ground singlet state, where all the electrons rotate in pairs in low-energy orbits. When irradiated with the wavelength of the PS absorption peak, one of the electrons in the highest occupied molecular orbitals of the PS moves to the lowest unoccupied molecular orbital, which places PS into a transient and unstable activated singlet state, leading to a series of events[29,30]. Due to the aromatic nature of many PSs, the energy difference between highest occupied molecular orbitals and lowest unoccupied molecular orbital is quite small. The light emitted at the excitation wavelength is usually in the visible or near-infrared parts of the spectrum. The activated singlet of the PS reverses the rotation of the activated electrons to generate a triplet, which is a process that enables triplets to cross between PS systems. The PS triplet has a lower energy and longer life than the singlet because the activated electrons spin parallel to the previous paired electrons and are not easily separated to create a singlet. To enter a more stable state, the triplet electron excited by the PS either enters the correct rotating orbit, is released to fall to the ground singlet state, and emits fluorescence (slow process) or directly interacts with molecules in the environment to return to the singlet state. The interaction between triplet states is reversible, but the interaction between the triplet state and the singlet state is irreversible. Therefore, the activated PS triplet can only interact with the molecules in the triplet rotation. In the cell, the triplet state of O_2 has obvious double radical properties in the ground state; therefore, energy is easily transferred from the triplet PS to oxygen molecules. This process is the basis of the chemical reaction of PDT.

The effectiveness of PDT is due to free radicals and electronically activated oxygen species, and their production depends on whether a PDT type I or II reaction occurs[31]

(Figure 1). As mentioned earlier, the outermost layer of the ground state triplet has two unpaired orbits that rotate in parallel; therefore, when the triplet state is excited by PS, the reactions of different molecules are quite different[29,32]. In a type I reaction, PS directly removes an electron to generate a superoxide anion (O_2^-). O_2^- can quickly form other substances, including hydroxyl radicals and hydrogen peroxide. In the type II reaction, the energy that excites the PS triplet state is transferred to O_2. The spin of the outermost electron of O_2 thus flips and moves to an orbit that previously contained unpaired (natural) electrons with opposite spins; this describes a singlet oxygen ($^1O_2$) in the active state. $^1O_2$ is not a free radical because all the electrons spin in pairs, but they are very active and short lived[33,34].

Generation of endogenous ROS

Under normal circumstances, approximately 90% of the ROS in the body are produced by the mitochondrial electron transfer chain[35]. The yield of ROS produced by the ETC increase during hypoxia, light stimulation, ischemia-reperfusion, aging, and mitochondrial respiratory depression. Over 90% of the oxygen in mitochondria is reduced by cytochrome oxidase to water molecules, while only 0.1%-0.2% of O_2 forms ROS through electron flow, mainly through electron transport chain complexes I and III[15,36]. The rate of the ROS produced by mitochondria is mainly affected by mitochondrial membrane potential (MMP) regulation.

In theory, PDT also has the ability to increase endogenous ROS by changing the MMP[37]. Generally, it is difficult for exogenous ROS to directly induce MMP changes to produce more endogenous ROS, but it can be accomplished through endoplasmic reticulum-mitochondria crosstalk. Briefly, PDT induces endoplasmic reticulum stress (ER stress, ERS), which mainly transmits death signals from the endoplasmic reticulum to the mitochondria by the proteinkinase R-like ER kinase (PERK) pathway. In this process, Ca^{2+} influx from the endoplasmic reticulum to mitochondria induces a decrease in the MMP and promotes ROS production[38,39]. In more detail, the mitochondria-associated ER membrane (MAM) may explain this phenomenon. MAMs whose contacting is increased in CSCs are connected by protein-binding complexes[40]. These two membranes are 10-25 nm apart and communicate through the calcium ion-related pathway[40]. Mitofusion2 (Mfn2) maintains MAM distance and prevents ER and mitochondria from being too close and thus prevent Ca^{2+}-mediated MMP changes and apoptosis. When 5-aminolevulinic acid mediated PDT is performed, Mfn2 expression was reduced, suggesting an production of endogenous ROS[41]. In addition, phosphofurin acidic cluster sorting protein 2 (PACS2) is an essential protein that mediates MAM-Ca^{2+} overload[42]. PACS2 promotes the cleavage of BCR-associated protein(BAP)-31, which is an important factor in the caspase-8 apoptosis pathway activated by Ca^{2+}. During PDT, BAP31 is cleaved before the mitochondria changes and mediates the release of ERS and Ca^{2+}[43]. From the point of view of enzyme catalysis, PDT regulates flavin adenine dinucleotide, ubiquinones, and cytochrome in mitochondrial respiratory chain complexes I and III to produce ROS[44-46]; mitochondrial triphosphopyridine nucleotide(NADPH) oxidase and xanthine oxidase catalyze the production of O^{2-}[47]; mitochondrial myeloperoxidase myeloperoxidase (MPO) catalyzes the production of OH; and protein kinase C catalyzes the production of H_2O_2[48,49]. Because the dynamic changes in ROS are rapid, in trace amounts, and complex, it is difficult to accurately detect the process by which ROS are produced, from exogenous to endogenous ROS, by PDT within a short period. Therefore, although we believe that PDT may induce endogenous ROS, very few studies have addressed or clarified this process.

Metabolism of ROS during PDT

According to the type of PDT photochemical reaction, ROS produced by subsequent metabolism can also be generally divided into two parts. The most active free radical molecule is •OH, which is converted into stable hydroxide ions by receiving electrons and generating water and protons. O_2^- receives electrons to form peroxide ions (O_2^{2-}) and then is rapidly protonated to form H_2O_2. O_2^- is inert in biological systems because the antioxidant action of superoxide dismutase converts O_2^- to H_2O_2 and O_2. H_2O_2 is converted into water and oxygen molecules[50,51]. Nonetheless, H_2O_2 may react with very low concentrations of electron-deficient substances[52], such as ferrous ions (Fe^{2+}), which cause the oxygen and oxygen bonds of H_2O_2 to break, producing ferric iron (Fe^{3+}), hydroxide, and •OH (Fenton reaction)[53]. •OH cannot be catabolized by enzymes but can be broken down by antioxidant peptides (such as glutathione) or antioxidant vitamins (such as vitamin C)[54]. PDT type I reaction products can also indirectly cause the formation of reactive nitrogen because O_2^- reacts with nitric oxide (NO) to generate peroxynitrite anion ($ONOO^-$)[17,55,56]. $ONOO^-$ is very active and has a

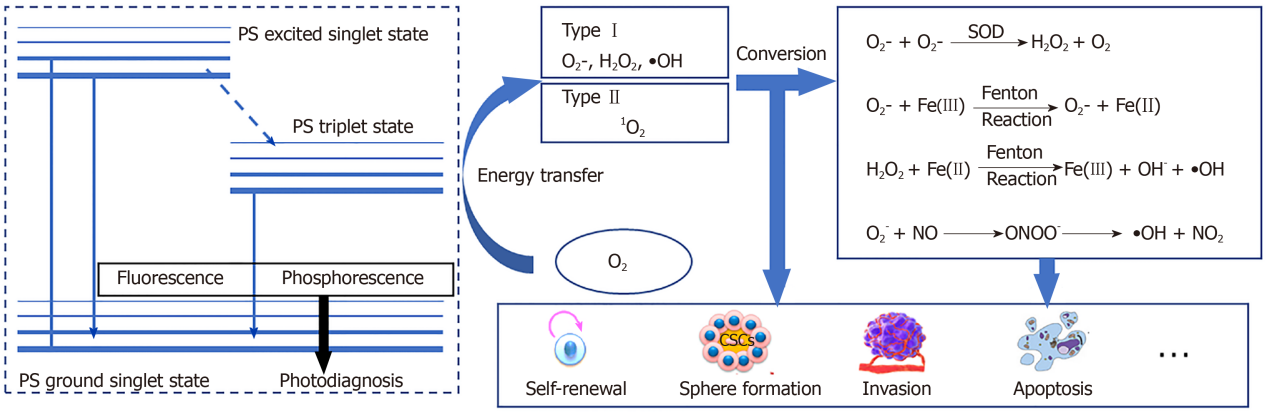

Figure 1 The process of reactive oxygen species production and transformation in cancer stem cells. A: The photosensitizer is activated from the ground singlet state to the excited singlet state and triplet state. The excited singlet state and triplet state can release energy through fluorescence and phosphorescence, or transfer energy to generate various reactive oxygen species (ROS); B: The mutual transformation of ROS *in vivo*. These ROS all affect the self-renewal, sphere formation, invasion, and apoptosis of cancer stem cells. PS: Photosensitizer; H_2O_2: Hydrogen peroxide; SOD: Superoxide dismutase; •OH: Hydroxyl radicals; OH^-: Hydroxyl; NO: Nitric oxide; $ONOO^-$: Peroxynitrite anion; NO_2: Nitrogen dioxide.

short life span. Rapid homogeneous fission forms •OH and nitrogen dioxide (NO_2). $ONOO^-$ also reacts with carbon dioxide to form carbonate anion radicals (CO^{3-}) and N O_2. All the resulting free radicals are destructive and they continue to move in the cell until they are paired (free-radical pairing). Although not a free radical, $^1O_2$ reacts with macromolecules in several different ways. $^1O_2$ can act as a dienophile in the Diels–Alder cycloaddition reaction, and it can react with the aromatics and the diene of the conjugated system, leading to the degradation of many lipids and proteins. Disulfide bonds and other electron-rich substances may also attack $^1O_2$[57] (Figure 1). In contrast to free radicals, $^1O_2$ cannot be destroyed by enzymes but can be inactivated by antioxidants (such as carotenoids).

IMPACT OF ROS FROM PDT ON CSCS

Location of photosensitizers in CSCs

The body has a complex antioxidant system. Normally, the production and removal of ROS are maintained in a dynamic balance so that they do not cause damage to the body[58]. Disruption of stem cell metabolism can directly determine whether stem cells are at rest, self-renewing, or differentiating[59-61], and controlling ROS levels is one of the feasible ways in which metabolism is disrupted. After PDT treatment, intracellular ROS accumulation increases, causing excessive oxidation of proteins, DNA, and lipids, which may be a direct means of controlling the fate of stem cells. ROS bind to proteins to generate carbonyl derivatives[62], alter the tertiary structure of the proteins, and promote protein/DNA[63]-protein cross-linking, leading to changes in the protein activity of CSC marker proteins such as octamer binding transcription factor 4 and sex determining region Y box 2 (Sox2)[64]. ROS directly attack DNA bases and easily cause deoxyguanosine modifications to one carbon atom (that is, 8-OHdG)[62], which may be one of the causes of point mutations in proto-oncogenes or tumor suppressor genes, such as Ras and p53[65,66]. Free radicals generate lipid peroxides through oxidation, which can damage cell membranes and promote ferroptosis.

However, whether ROS oxidize lipids, inactivate proteins, or damage DNA in CSCs largely depends on where the ROS are generated (because the half-life of the ROS produced by PDT ranges from 3.5 μs to 5 s, and the $^1O_2$ diffusion distance is approximately 40 nm)[14]. Some people think that the killing effect of a PS in mitochondria is significantly higher than that of the same PS is in other organelles, and the importance of PS positioning is even higher than the ROS yield[67]. Therefore, before describing the effects of PDT and ROS on CSCs in detail, it is necessary to summarize the subcellular localization of commonly, recently used PSs. Of course, in special cases, PDT undergoes different localization and interacts with unique effector sites before activation. For example, in our previous studies, we observed that a mitochondrial PS induced ERS-mediated apoptosis, which suggested that intercellular organelle crosstalk was involved in the ROS-regulated CSCs[68]. In this review, PS

mapping for tumor PDT can be roughly divided into three areas: Mitochondria[69-78], endoplasmic reticulum[12,68,79-82], and lysosomes[83-88]. These PSs can be roughly summarized into porphyrin-based photosensitizers, such as porphyrins[89-92], chlorins[80,93,94], phthalocyanines, and naphthalocyanines[95-99], and non-porphyrin-based photosensitizers, such as cyanine, methylene blue, Nile blue, rhodamine, triarylmethane, and acridine[68,100-105]. These PSs and their derivatives are summarized by category in Table 1. Therefore, to more accurately control and eliminate CSCs, the effects of ROS on PDT in different cell structures should be classified and explained.

By the way, it is worth mentioning that photochemical internalization is a special CSC targeting strategy though PDT does not play a major role in this approach. In photochemical internalization, PS are modified by CSC biomarkers (such as CD133, CD44, CSPG4, and EpCAM)[106-109] that are first anchored to the cell membrane and then are endocytosed into intracellular vesicles. Finally, the drugs carried into the cell are released through PDT. For more details, please refer to a previous review[110].

ROS and mitochondria in CSCs

The effect of ROS on the function of mitochondria has always been one of the research focuses of CSCs. ROS produced through PDT can induce apoptosis through increased mitochondrial membrane potential[111]. Studies have shown that mitochondrial photooxidative stress can cause a large number of lipid peroxidation reactions in the mitochondrial membrane, leading to rapid changes in the MMP, which stimulates pressure-dependent anion channels [voltage-dependent anion channels (VDACs)][112] and promotes the opening of the permeability transition pore complex[113,114], thereby releasing cytochrome C (Cyt C)[115,116] into the cytoplasm. Cyt C combines with caspase-1 to form a multimer, which initiates apoptosis in CSCs in a caspase-dependent manner; in the non-caspase-dependent apoptosis pathway, other proteins are activated and released, such as apoptosis inducing factor, Omi/HtrA2, and endonuclease G during PDT[117-119]. The release of these enzymes depends on the cleavage of calpain[120]. These factors all directly lead to the apoptosis of CSCs.

B-cell leukemia 2 (BCL-2) family protein interactions are important in the induction of apoptosis induced by changes to the MMP in CSCs. And they are also important mitoROS modulators. In the canonical pathway, BCL-2 associated X and K proteins are located on the cytoplasmic side and the mitochondrial side of the outer mitochondrial membrane under normal conditions, respectively. When the MMP is increased, BCL-2 associated X protein is transported and inserted into the outer mitochondrial membrane, and the local conformation of BCL-2 associated K protein forms homo-oligomers or hetero-oligomers, which release Cyt C in the membrane space and initiate CSC apoptosis[121]. In the non-canonical pathway, BCL-2 may be involved in the regulation of the cell redox state without antioxidant characteristics. First, there is a physical interaction between BCL-2 and CcOVα (cytochrome c oxidase subunit Vα). Overexpression of BCL-2 causes an increase in mitochondrial localized CcOVα, which is conducive to CcO total enzyme assembly and the ETC process[122,123]. Second, the BCL-2 BH3 domain interacts with glutathione (GSH) *in vitro*, suggesting that BCL-2 functions in regulating mitochondrial GSH content[124]. Finally, the mitochondrial localization of GTPase-Rac1, which is associated with stem cell deletion, and its interaction with BCL-2 suggest that Rac1 plays an important antioxidant role[125]. These results suggest that BCL-2 may be a bridge connecting mitochondrial apoptosis and ROS in CSCs.

Energy metabolism is one of the main functions of mitochondria, and this process is closely related to the stemness of tumor cells. Research on energy metabolism in CSCs at various stages is rife with controversy. Early studies found that CSCs have more obvious anaerobic glycolytic characteristics than are expressed in differentiated cancer cells; that is, CSCs have increased expression of glycolytic enzymes, increased production of lactic acid, and decreased or resting mitochondrial function[126,127], and their ROS levels are usually lower than those of cancer cells. Recent studies have suggested that mitochondria in CSCs have increased mass and membrane potential, and their mitochondrial function reflects higher mitochondrial ROS levels and enhanced oxygen consumption rates[128]. In any case, mitochondrial function and oxygen concentration are essential for maintaining CSC function[129,130]. It has been inferred that under hypoxic conditions, some CSCs preferentially undertake oxidative phosphorylation for survival and maintenance of stemness and convert to glycolytic metabolism during differentiation[131]. The production of ROS by PDT consumes a large amount of oxygen, which forces CSCs to change from oxidative phosphorylation in the "stem cell" state to anaerobic glycolysis during differentiation, suggesting that PDT can effectively control the differentiation of CSCs. In addition, ROS can reduce the expression of caveolin-1 in cancer-associated fibroblasts, the major component of

Table 1 Subcellular localization of photosensitizers in different cancer stem cells

Photosensitizer	Location	Cancer (parental cell lines)	Ref.
5-aminolevulinic acid	Mitochondria	Breast cancer; T-cell leukemia	Song *et al*[89] Yang *et al*[90]
Protoporphyrin IX	Mitochondria	Colorectal cancer (HT29); liver cancer (Hep3B)	Taba *et al*[91]
Porphyrin-based photosensitizer meso-5-[ρ-diethylene triamine pentaacetic acid- aminophenyl]-10,15,20-triphenyl-porphyrin	Lysosome	Gastric cancer (HGC27, SNU-1)	Chen *et al*[92]
Porphyrin-based amphiphilic block copolymer (PEG-b-PCL-a-porphyrin)	Nucleus; lysosome	Lung cancer (A549)	Tian *et al*[83]
Tetrahydroporphyrin-tetratosylat	Lysosome	Human bronchial smooth muscle cells (HBSMCs)	Berndt-Paetz *et al*[85]
Meso-5-[ρ-diethylene triamine pentaacetic acid- aminophenyl]-10,15,20-triphenyl-porphyrin	Lysosome	Gastric cancer (HGC27, SNU-1)	Chen *et al*[92]
{Carboxymethyl-[2-(carboxymethyl-{[4-(10,15,20-triphenylporphyrin-5-yl)-phenylcarbamoyl]-methyl}-amino)-ethyl]-amino}-acetic acid	Lysosome	Liver cancer (HepG2); gastric cancer (BGC-823)	Chen *et al*[88]
Sinoporphyrin sodium (DVDMS-2)	Endoplasmic reticulum	Cholangiocarcinoma (CX-1)	Kong *et al*[12]
Chlorin e6	Mitochondria; cytoskeleton; lysosome; endoplasmic reticulum	Gliosarcoma (9L/LacZ); colorectal cancer (SW620)	de Almeida *et al*[93] Yang *et al*[80]
Meta-tetrahydroxyphenylchlorin	Mitochondria, endoplasmic reticulum	Colorectal cancer (SW620)	Abdulrehman *et al*[81]
β-CD through an amide bond	Mitochondria	Breast cancer (MCF-7)	Tong *et al*[94]
Palmatine hydrochloride	Mitochondria, endoplasmic reticulum	Breast cancer (MCF-7); colorectal cancer (HT29)	Wu *et al*[77] Wu *et al*[78]
Ce6-loaded branched polyethylenimine-PEGylated ceria nanoparticles	Lysosome; large vesicle	Cervical cancer (Hela)	Yang *et al*[86]
Mannose-conjugated chlorin	Lysosome; endoplasmic reticulum	Tumor-associated macrophages (TAM)	Hayashi *et al*[82]
Glucose-conjugated chlorin compound e6	Endoplasmic reticulum	Esophageal cancer (OE21,KYSE30); gastric cancer(MKN45); colorectal cancer (HT29)	Ichikawa *et al*[79]
(G-Mito-Pc) silicon (II) phthalocyanine (Pc)	Mitochondria	Cervical cancer (Hela)	Zhao *et al*[95]
Zinc (II) phthalocyanine	Mitochondria; lysosome	Liver cancer (HepG2)	Chen *et al*[96]
Chloroaluminum phthalocyanine	Mitochondria	Prostate cancer (LNCaP)	Leandro *et al*[97]
Pheophorbide a	Mitochondria	Cervical cancer (Hela)	Choi *et al*[98]
Silicon (IV) phthalocyanines	Mitochondria	Liver cancer (HepG2); gastric cancer (BGC-823)	Zheng *et al*[99]
Tamoxifen-zinc (II) phthalocyanine	Lysosome	Breast cancer (MCF-7)	Zhang *et al*[84]
Cyanines (AlPc and Pc green)	Lysosome	Gastric cancer (EPG85-257P)	Zielichowska *et al*[87]
Cyanine-derivative photosensitizer	Mitochondria	Breast cancer (MCF-7)	Zhao *et al*[69]
Ru(ii) polypyridyl complexes	Mitochondria; lysosome	Lung cancer (A549)	Qiu *et al*[100]
Ethyl acetate extract of cichorium	Mitochondria	Colorectal cancer (SW620, HCT116)	Wen *et al*[68]
Hypocrellin A	Mitochondria	Lung cancer (A549)	Qi *et al*[101]
Cyanopyridinium salts	Mitochondria	Cervical cancer (Hela)	Zhao *et al*[69]
Rhodamine organic dye	Mitochondria	Breast cancer (MCF-7)	Liu *et al*[70]
Cis-[Pt(NH)(L)Cl](NO) (1-3) having boron-dipyrromethene (BODIPY) pendants (L) with 1,3,5,7-tetramethyl-8-(4-pyridyl)-4,4'-difluoroboradiazaindacene moieties	Mitochondria	Lung cancer (A549); breast cancer (MCF-7)	Raza *et al*[72]
Hypericin	Mitochondria	Adenoidal thyroid cancer (FRO)	Kim *et al*[102]

Platinum(II) complexes [Pt(L)(R-BODIPY)]Cl	Mitochondria	Lung cancer (A549); breast cancer (MCF-7); cervical cancer (Hela)	Ramu *et al*[103]
Cis-[Pt(NH)(L)Cl](NO), where L is an imidazole base conjugated to 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY)	Mitochondria	Breast cancer (MCF-7)	Raza *et al*[72]
Triphenylphosphonium	Mitochondria	Lung cancer (A549)	Rui *et al*[74]
Triphenylphosphonium	Mitochondria	Cervical cancer (Hela)	Choi *et al*[98]
Methyl-functionalized derivatives of the drug carrier triphenylphosphonium	Mitochondria	Cervical cancer (Hela); gastric cancer (FU97)	Hu *et al*[75]
Hypocrellin B	Mitochondria	Breast cancer (MDA-MB-231)	Jia *et al*[76]
Aloe-emodin	Mitochondria; endoplasmic reticulum	Osteosarcoma (MG63)	Li *et al*[104]
BODIPY-Appended 2-(2-Pyridyl) benzimidazole Platinum (II) Catecholates	Mitochondria	Keratinocytes (Hacat)	Mitra *et al*[105]
Acetylatedglucose-conjugated chlorin	Endoplasmic ruticulum	Esophageal cancer (OE21, KYSE30); gastric cancer (MKN45);	Ichikawa *et al*[79]

tumor stroma. The reduction of caveolin-1 stabilizes HIF-1α (which forms a heterodimer), enhances glycolysis to adapt to hypoxic conditions, and leads to a further increase in ROS production[132-134]. These studies indicate that oxygen depletion in mitochondria caused by PDT mediated ROS production inhibits stemness of cancer cells.

Some key genes may mediate both of these effects. PDT inhibits the Wnt pathway[135] which plays an important role in CSCs[136,137], inducing mitochondrial repression and glycolytic conversion by activating Dlx-2 and Snail[138-140]. This mitochondrial suppression is mediated by the inhibition of mitochondrial complex IV[140]. Wnt also directly targets pyruvate dehydrogenase kinase, thereby inhibiting mitochondrial respiration and promoting glycolytic conversion[141]. Therefore, the Wnt pathway may regulate CSCs through the above two functions at the same time. Currently, little is known about the relationship between typical stemness markers and the regulation of CSC metabolism, but researchers have shown that the stem cell marker CD44 may be crucial in the regulation of glycolytic metabolism[142,143]. The direct interaction between CD44 and the GSH transporter--solute carrier family 7 member 11 has been reported in multiple PDT articles[144,145], also suggesting the ability of PDT to manipulate CSCs through redox and energy metabolism.

Mitochondrial autophagy also plays a protective role against ROS in CSCs. Currently, it is widely recognized that mitochondrial autophagy counteracts PDT. For details, please refer to our previous review[146].

ROS and the endoplasmic reticulum in CSCs

The most widespread and common effect of PDT-mediated photooxidative stress is the unfolded protein response, secondary to endoplasmic reticulum stress (UPR^{ER}, UPR). Increased endoplasmic reticulum-related ROS in PDT have been shown to cause upregulation of various ER molecular chaperones, such as calcium-binding proteins (GRP78/Bip and GRP94) and protein disulfide isomerase. These key proteins lead to the accumulation of unfolded proteins in the ER cavity, leading to PERK-, IRE1-, and ATF6-mediated UPR[147-149]. In the intestine, activation of UPR by PERK kinase leads to differentiation of intestinal epithelial stem cells and colon CSCs, and the absence of X box binding protein 1 results in increased stemness and adenoma formation. X box binding protein 1 activation results in reduced cell proliferation and intestinal epithelial cell stemness due to cross-activation of the PERK-eIF2α signaling[150]. In pancreatic cancer, GRP78 downregulates stem cell clone formation and self-renewal characteristics, suggesting that the UPR plays a role in inhibiting stemness in pancreatic cancer[151]. The contradictory results mentioned above can be explained by the dual nature of UPR^{ER} in both survival and apoptosis signaling. The fate of cells with respect to these two signal cascades depends on the intensity of the photooxidative stress. Severe ER photooxidative stress can stimulate more cascades that are transducing death-promoting signals (such as apoptosis), such as that stimulated by CCAAT/enhancer binding protein homologous protein, which is a key pro-apoptotic transcription factor in ERS[152,153]. A small or low level of ER photooxidative stress can stimulate more promoting survival signaling cascades (such as autophagy, p38 mitogen-activated protein kinase(MAPK) signaling, antioxidant

signaling, and amino-terminal kinase JNK signaling)[154,155].

In addition to the UPR mechanism described above, ROS can affect the fate of CSCs through endoplasmic reticulum-mitochondrial crosstalk. PDT treatment causes the release of internal Ca^{2+} of the endoplasmic reticulum into the cytoplasm and mitochondria, inducing MMP-mediated apoptosis. In the above process, MAM (mitochondria-associated membrane), which is a solid-state connection between mitochondria and the endoplasmic reticulum, overexpress in CSCs. Its state and efficiency of the coupling are among the primary regulatory characteristics by which factors influence Ca^{2+} concentration in mitochondria[38-40]. Therefore, ROS generated by PDT may affect MAMs by various genes, such as P53, PML, ERO1, and p66Shc. P53 proved to be differentially expressed in PDT is involved in the regulation of Ca^{2+}-mediated apoptosis in a transcription-independent manner. Among the proteins that accumulate in the MAMs, p53 activates the pathway to cell death, while p53 deletion leads to a Ca^{2+} decrease in the endoplasmic reticulum[156]. PML (promyelocytic leukemia) proved to be another tumor suppressor involved in regulating the endoplasmic reticulum-mitochondrial Ca^{2+} dialog[157]. The endoplasmic reticulum oxide protein endoplasmic oxidoreductin 1(ERO1)-Lα regulates the release of Ca^{2+}, and the generation of ROS, from the endoplasmic reticulum through inositol 1,4,5-triphosphate receptor type 1 and thioredoxin domain containing 4[158,159]. It is involved in the formation of disulfide bonds with protein disulfide isomerase and therefore plays an important role in protein folding. p66Shc is an ROS-generating protein located in MAMs. When it undergoes oxidative stress, p66Shc is phosphorylated at Ser36 and then is translocated to mitochondria and/or MAMs. It is involved in ROS generation and apoptosis-related signaling pathways[160,161].

By integrating some studies, we also speculate that some factors are involved in the regulation of ERS and MAM at the same time after PDT treatment. First, PERK is highly expressed after PDT and can activate UPR. PERK is found to be localized to MAMs and promotes endoplasmic reticulum-mitochondria coupling[162]. Second, Mfn2, a kind of skeleton in MAMs, can regulate the endoplasmic reticulum associated autophagy and apoptosis by downregulating the activity of PERK[163,164]. PACS2, another important component of MAM, also participates in the autophagy process. In PACS2-knockout and Mfn2-knockout cells, the accumulation of autophagic markers and the translocation of endoplasmic reticulum-related proteins were significantly reduced, indicating that MAMs play specific roles in the formation of autophagosomes and ERS[165]. This shows that there is mutual regulation between ERS and MAM. Based on the research of ER-mitochondria crosstalk, the efficacy of autophagy and ERS inhibitors/activators combined with PDT in the treatment of CSCs has been verified in multiple studies, regardless of whether the photosensitizer is localized to the endoplasmic reticulum or mitochondria.

ROS and lysosomes in CSCs

Lysosome status also directly or indirectly affects CSCs, such as through apoptosis initiation or autophagy flux. However, in addition to apoptosis and autophagy, lysosomes have recently been found to an play important role in the switch of eukaryotic cells to deep quiescence[166]. The dormant state that can be reversed by the stimulation of growth signals is called cell resting. In contrast, it is irreversible in state of senescence. The depth of the resting state of the cells is directly related to the difficulty of maintaining the stemness of CSCs and re-entering the proliferative state. Fujimaki *et al*[166] found that resting depth-related genes were significantly enriched in the lysosome pathway. By measuring the autophagy flux that characterizes lysosomal function, it was found that, as the resting state of the cells deepened, lysosomal function gradually decreased. The exogenously expressed transcription factor microphthalmia associated transcription factor enhanced lysosomal function in cells, and an increase in lysosomal function reduced the concentration of the ROS in the cell to prevent deepening of the resting state. The "switch" works by regulating the concentration of ROS in cells. The ROS produced by PDT may also regulate the resting state of cells through lysosomes, which may interfere with the stemness of CSCs and play a therapeutic role.

PDT mediates the activation of lysosomal-related PS, which can significantly induce the production of autophagy and mediate the release of cathepsin and lysosomal-mitochondrial crosstalk. The proliferation of colonic CSC spheres depends on the key autophagy related mTORC kinase, which is activated by the ROS produced by the NADPH oxidase (NOX)[167]. NOX1 is colocalized with mTORC1 in vacuolar assembly protein (VPS)41-/VPS39- lysosomes, where mTORC1 binds S100A9 (a member of the S100 calcium-binding protein) in an ROS-dependent manner, and S100A9 can thus be oxidized by ROS. This finding indicates that ROS in VPS41-/VPS39- lysosomes

mediate S100A9 oxidation and mTORC1 activation, which are essential processes for colonic CSC proliferation and colon cancer progression[168]. Moreover, NOX1/2 and ROS-containing endosomal compartment co-localize with RAS and associated protein (RAB) 5/7[169,170], which is involved in the lysosomal-mitochondrial crosstalk pathway and plays a key role in maintaining CSC survival[171]. The investigators determined that RAB5/7 overexpression can effectively inhibit CSCs. Further, they found that mefloquine hydrochloride (an autophagy inhibitor) is involved in the endolysosomal pathway by targeting RAB5/7, and this effect is dependent on the mitochondrial autophagy key protein PTEN induced putative kinase 1/parkinson disease protein 2. Although there is no direct evidence, it can be speculated that ROS produced by NOX may mediate lysosomal-mitochondrial crosstalk.

Fe^{2+} plays an important role in lysosomal-mitochondrial crosstalk. The substance to be degraded in the cytoplasm is entrapped in autophagic vesicles, and then, the contents of autophagic vesicles and lysosomes are degraded under the action of lysosomal enzymes. Autophagy leads to a large amount of redox-active iron (Fe^{2+}) accumulating in the lysosome cavity. These high concentrations of active iron can cause membrane instability when the lysosome is undergoing slight oxidative stress. PDT that targets lysosomes can directly result in the release of a large number of proteases upon photooxidative stress and thus promote activation of endogenous apoptosis-related protein[172,173]. In addition, in the study of the antitumor effect of bafilomycin (an autophagy inhibitor) combined with phthalocyanine 4 (Pc4, photosensitizer), the lysosome was found to be alkalized by bafilomycin, causing the collapse of the pH gradient and the release of Fe^{2+}. Subsequently, mitochondria accumulate a large amount of active iron (Fe^{2+}) through the one-way Ca^{2+} transport channel, thus generating OH through the Fenton reaction with H_2O_2. Thus, the apoptosis pathway is initiated. This study shows that autophagy inhibitors combined with Fe^{2+} can further increase the effect of PDT on CSCs. In fact, the Fenton reaction is very commonly used in the design and synthesis of nano-photosensitizers. Currently, some studies have used the Fenton reaction to increase the concentration of O_2[174] or hydroxyl radicals[175] in the tumor microenvironment, and others used it to directly kill CSCs. PDT combined with the Fenton reaction[176] is a feasible treatment method for CSCs showing strong drug resistance. In addition, a new type of programmed cell death, ferroptosis, is also closely related to Fe^{2+} and the Fenton reaction[177]. Some researchers have found that ferricin chelates lysosomal iron[178]. These compounds induce iron depletion by preventing iron transport, leading to the dissolution of ferritin. Enzymatic degradation, followed by ROS formation and iron release, mediates the lysosomal ferroptosis pathway. In the development of PS, some scientists have induced ferroptosis by artificially reducing Fe^{2+} or releasing Fe^{2+} in the lysosome, creating a potential therapeutic mechanism for killing apoptosis-resistant CSCs (Figure 2).

ROS and cell membranes in CSCs

Excessive lipophilic dyes, anionic dyes, and photosensitizers (especially localized on biological membranes) can affect different unsaturated phospholipids and membrane cholesterol, leading to excessive lipid peroxidation[179], which directly leads to a wide range of membrane changes, loss of membrane integrity and fluidity, and inactivation of the membrane protein system, causing accidental cell necrosis. This effect can be induced with all types of PDT under sufficient ROS. The exogenous pathway of apoptosis is mediated by death receptors on the cell membrane. Tumor necrosis factor type-I receptor (TNFR1), Fas/CD95, DR3, and TNF-related apoptosis-inducing ligands R1 and R2 (TRAIL-R1 and TRAIL-R2) were found to be differentially expressed upon PDT, and their death domains are necessary for exogenous apoptosis. Exogenous apoptosis induced by PDT is usually activated by cytokines released by photooxidative stress or dead cells[180,181]. A very low dose of sulfathiophene (2.0 μg/mL) combined with radachlorin-PDT (0.5 μg/mL) showed a synergistic effect of exogenous and endogenous apoptosis[182]. The CSCs in the CD44 (+)/CD24 (-/low) subgroup were more sensitive to Fas- and TRAIL-mediated exogenous apoptotic pathways; therefore, the PDT-induced exogenous receptor apoptosis pathway may be very sensitive in this subgroup[183]. Through methotrexate-mediated epigenetic enhancement of ALA-PDT, Salva and colleagues restored sensitivity to death receptor-related pathways and increased the effect in subgroups of cells with low expression of Fas[184]. Exogenous and endogenous apoptosis induced by PDT generally does not happen separately. In many studies, PDT mediated by phenalenone[181], coralyne[185], ALA[186], hypericin[187], dipyrromethene boron difluoride(BODIPY)[188], and zinc phthalocyanine[189] can cause both endogenous and exogenous apoptosis. This dual apoptosis phenomenon is generated primarily by signaling molecules, including p38

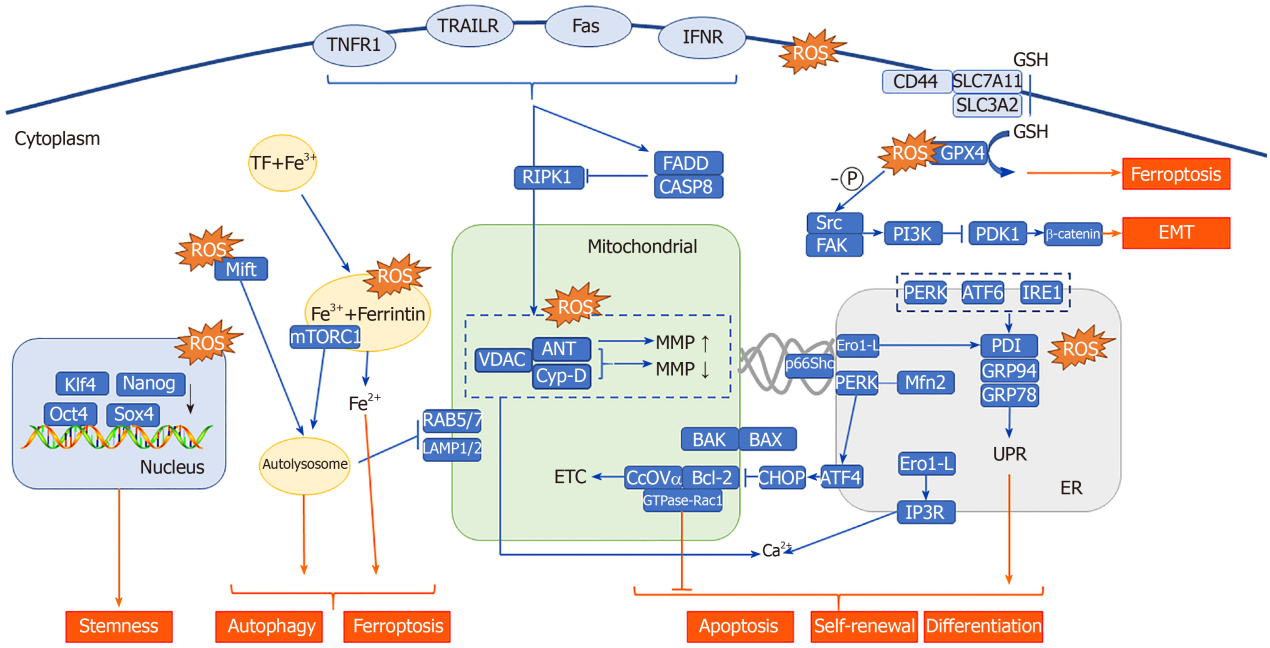

Figure 2 Reactive oxygen species located in different subcellular structures affect the stemness, self-renewal, differentiation, apoptosis, autophagy, ferroptosis, and epithelial-mesenchymal transition of cancer stem cells through a variety of molecules. TNFR1: Recombinant human tumor necrosis factor receptor type 1; TRAILR: TNF-related apoptosis-inducing ligand; IFNR: Interferon Receptor; ROS: Reactive oxygen species; CD44: Cluster of differentiation 44; SLC3A2: Solute carrier family 3 member 2; SLC7A11: Solute carrier family 7 member 11; GSH/GSSG: Glutathione; GPX4: Glutathione peroxidase 4; EMT: Epithelial-mesenchymal transition; TF: Transferrin; RIPK1: Receptor interacting protein kinase 1; FADD: Fas-associating protein with a novel death domain; CASP8: Caspase 8; Mitf: Melanocyte inducing transcription factor; mTORC1: Mammalian target of rapamycin complex 1; VDAC: Voltage-dependent anion-selective channel; ANT: Adenine nucleotide translocator; MMP: Mitochondrial membrane potential; ER: Endoplasmic reticulum; MAM: Mitochondria-associated ER membrane; Ero1-L: Endoplasmic reticulum oxidoreductin-1-L; PERK: Protein kinase RNA-like endoplasmic reticulum kinase; ATF4/6: Activating transcription factor-6; IRE1: Inositol-requiring enzyme 1; Mfn2: Mitofusin-2; PDI: Polydispersity index; GRP94/78: Glucose-regulated protein94/78; Klf4: Krüppel-like factor4; Nanog: Homeobox protein NANOG; Oct4: Octamer-binding protein 4; Sox2: SRY-box 2; LAMP1/2: Lysosomal associated membrane protein 1/2; ETC: Electron transport chain; BAK: Bcl-2 homologous antagonist/killer; BAX: Bcl-2-associated X protein; CHOP: C/EBP-homologous protein antibody; CcOVα: Cytochrome c oxidase subunit Vα; Rac-1: Ras-related C3 botulinum toxin substrate 1; IP3R: Inositol 1,4,5-trisphosphate receptor; UPR: Unfolded protein response.

MAPK, Janus kinase (JAK)-2, and signal transducer and activator of transcription(STAT)-1.

ROS-induced immunogenicity and CSCs

PDT is a treatment method that mainly induces apoptosis, and most forms of apoptosis are based on immune silencing or immune tolerance, that is, tolerogenic apoptosis[190]. This immune tolerance to apoptosis is an important host protection mechanism. After photooxidative stress, most apoptotic cells express chemotaxis signals, such as intercellular cell adhesion molecule, phosphatidylethanolamine, phosphatidylinositol, and low-density lipoprotein, and signals that inhibit anti-phagocytosis proteins, such as CD31 and CD47, to promote the phagocytosis of tolerogenic cells by macrophages, prevent immune responses, and exhibit tolerant immunobiological characteristics[191-194].

However, in a significant portion of PDT samples, lysates of necrotic or apoptotic cells enhance immunogenicity[173]. Through this PDT-induced enhanced immunogenicity, CSCs may be cleared by immunocytes. Immunogenic apoptosis has all the biochemical markers of tolerogenic apoptosis, but tolerogenic apoptosis does not have the following two main characteristics: (1) Exposure to or secretion of important immunogenic signaling proteins or damage-associated molecular patterns (DAMPs); and (2) The ability to activate the host's immune system. DAMPs can be secreted or expressed on the cell surface after cell injury or death to promote inflammation[195,196]. It has been found that photooxidative stress, especially mitochondrial photooxidative stress, can cause DAMPs to be expressed on the cell surface (ecto-) or released outside the cell (exo-), such as calreticulin (CRT) and GRP78[197,198]. IFN-1 has recently been recognized as a new type of DAMP that links innate and adaptive immunity, and it has been hypothesized to be the basic

requirement for inducing immunogenic cell death, especially through the activation of dendritic cells. Me-ALA-induced PpIX (endogenous PS) was found to upregulate IFN-1 expression in B16-OVA melanoma cells[199]. This upregulation of α/β transcripts coincided with the interferon regulatory factor-3 phosphorylation that activated STAT1 and increased the expression of ligand receptors and interferon- stimulated genes (ISGs, like chemokine (C-X-C motif) ligand 10, interferon-induced GTP-binding protein Mx1, and ubiquitin-like protein ISG15). In this sense, PDT-treated melanoma cells induce IFN-1-dependent phenotype maturation of dendritic cells by enhancing costimulatory signals (CD80 and MHC-II molecules) and tumor-directed chemotaxis. Based on the discovery of enhanced immunogenicity, there have been studies combining PDT and immunotherapy, loading si-PD-L1[200], docetaxel (DTX)[201], PD-L1 monoclonal antibody[202], and anti-PD-L1 peptide[203], among others, to enhance the antitumor effect.

Compared with those of specific drug-induced immunogenicity, PDT-mediated immunogenic activation also has certain targeting advantages. Although mitoxantrone, mitomycin C, 5-fluorouracil, camptothecin, cisplatin, oxaliplatin, ultraviolet radiation, and γ-radiation can induce ROS-mediated ERS[204-207], in most cases, ERS induction is nontargeted and not completely efficient. In the pre-apoptotic stage, ERS-mediated immunogenic apoptosis is accompanied by ecto-CRT and exo-ATP production[197,201]. Another study found that Photofrin-PDT mainly produces mitochondrial photooxidative stress and a small amount of ER photooxidative stress. ER photooxidative stress mediated by hypericin-PDT can induce immunogenic apoptosis through targeted oxidative stress[208]. The expression of pre-apoptotic ecto-CRT in the mitochondria of Hyp-PDT-treated cells is not as high as it is under ERS targeting[197]. This finding suggests that photosensitizers targeting the ER may have a better effect in inducing immunogenicity.

ROS-mediated EMT in CSCs

The activation of cancer cell invasion and metastasis is one of the characteristics that changes the normal function of cells to acquire enhanced malignant growth, and it is also the main obstacle for humans to overcome cancer[209]. The EMT refers to the biological process in which epithelial cells are transformed into cells with interstitial phenotypes under special physiological or pathological conditions; that is, the epithelial cells lose their original phenotype connected with the basement membrane and acquire resistance to the phenotype of interstitial cells, such as the ability to undergo apoptosis or degrade the extracellular matrix, and higher migration and invasion[210]. The weakening of tumor cell adhesion and the enhancement of tumor cell movement are the basis of invasion and metastasis. The EMT provides the conditions for invasion and metastasis of tumor cells of epithelial origin. Therefore, the EMT is closely related to tumor invasion and metastasis. The relevant molecular mechanism of the EMT in tumor cells is the loss of cell epithelial morphology and related markers (including E-cadherin, desmosome smoothelin, Muc-1, cytokeratin 18, occludins, claudins, and ZO-1) and the acquisition of mesenchymal markers (including N-cadherin, vimentin, fibronectin, vitronectin, alpha smooth muscle actin [α-SMA], and FSP1)[27]. This process is affected by the regulatory effects of Snail, Slug, ZEB1, and Twist1 in the tumor microenvironment. Among all the factors that promote tumor cell migration, ROS play key roles by activating signals that cause cell migration, such as activating the proto-oncogene tyrosine protein kinase (Src) and focal adhesion kinase (FAK)[211]. The EMT is the initial step of ROS-activated tumor cell migration. During the ROS generation process, the EMT is also affected by the regulation of Src and FAK, resulting in tumor cell migration. Studies have found that para-cresyl sulfate can promote tumor cell migration by activating ROS/Src/FAK signaling in bladder cancer tumor cells[212]. Goulet *et al*[213,214] found that IL6 can regulate cancer-associated fibroblast-induced EMT through the STAT3/AKT signaling pathway in bladder cancer, induce myeloma cell proliferation through Src, and regulate the Src/STAT3 signaling pathway in lymphatic endothelial cells. Therefore, IL6 may be a regulator of ROS-activated Src/FAK signaling.

The EMT plays an important role in the process of acquiring stemness by tumor stem cells. Transcription factors that regulate EMT, such as Snail, ZEB1, and Twist1, are involved in the process of conferring stemness to CSCs. For example, Snail can induce a CSC phenotype in colorectal cancer cells, in which it enhances stemness properties and resistance to radiotherapy[215]. ZEB1 inhibits the expression of miRNAs, including miR-183, miR-200c, and miR-203, and thus upregulates the stem cell-related factors Sox2 and Klf4. Knocking out ZEB1 prevents not only the EMT and cell invasion and metastasis but also the emergence of stem cell phenotypes[216]. Twist1 can induce the EMT and stem cell properties by increasing Bmi-1 expression and acting

synergistically with it. EMT induced by TGF-β1 plays a key role in the generation of CSCs and is involved in maintaining the characteristics of CSCs, such as self-renewal and differentiation[217]. Most cholangiocarcinoma CSCs have epithelial and mesenchymal characteristics and express EMT markers. IL6, which may be involved in ROS-activated Src/FAK signaling, can regulate stem cell self-renewal. In breast cancer, head and neck squamous cell carcinoma, gastric cancer, and glioma, IL-6 promotes stem cell self-renewal through the classic IL-6R/gp130/STAT3 signaling pathway, while IL-6 also increases N-cadherin, E-cadherin, Twist, Snail, and Vimentin expression to accelerate the tumor cell EMT, which leads to cancer metastasis. Studies have shown that ROS may activate the NF-κB pathway and cause gastric cancer cells and cancer-associated fibroblast cells to release IL-6, thereby mediating tumor metastasis and CSC self-renewal and maintenance[218,219]. It can be seen from the above literature that PDT mainly inhibits CSCs through IL-6/SRC/FAK mediated EMT mechanism.

CONCLUSION

Although PDT is promising in the treatment of CSCs, several pitfalls will have to be overcome in order to take a step forward in clinical application. First, despite the obvious tumor targeting of PS, the heterogeneity of CSCs determines that it is difficult for a single targeted PS to eliminate all CSCs. The single-cell sequencing will be very useful for exploring molecular subtypes common to different CSCs. Second, CSCs account for a small proportion of tumors and are widely distributed. In order to enhance the killing effect against deep CSCs by PDT, it is necessary to design the PS that can absorb longer wavelengths. The dosimetry of PDT is also one of the existing challenges. The ROS yield of PS determines whether the CSCs will metastasize and recur. Developing different PS-PDT treatment criteria from different subcellular localizations can help reduce adverse outcomes. Finally, in view of the immune activating effect of PDT, PDT combined with immunotherapy has shown good experimental results. However, how to safely and effectively use PDT to activate immunity is still a difficult problem in clinical treatment design. Based on these considerations, although PDT exhibits anti-tumor (including CSCs) effects, further research is needed to expand clinical application.

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REVIEW

Decellularized adipose matrix provides an inductive microenvironment for stem cells in tissue regeneration

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Abstract

Stem cells play a key role in tissue regeneration due to their self-renewal and multidirectional differentiation, which are continuously regulated by signals from the extracellular matrix (ECM) microenvironment. Therefore, the unique biological and physical characteristics of the ECM are important determinants of stem cell behavior. Although the acellular ECM of specific tissues and organs (such as the skin, heart, cartilage, and lung) can mimic the natural microenvironment required for stem cell differentiation, the lack of donor sources restricts their development. With the rapid development of adipose tissue engineering, decellularized adipose matrix (DAM) has attracted much attention due to its wide range of sources and good regeneration capacity. Protocols for DAM preparation involve various physical, chemical, and biological methods. Different combinations of these methods may have different impacts on the structure and composition of DAM, which in turn interfere with the growth and differentiation of stem cells. This is a narrative review about DAM. We summarize the methods for decellularizing and sterilizing adipose tissue, and the impact of these methods on the biological and physical properties of DAM. In addition, we also analyze the application of different forms of DAM with or without stem cells in tissue regeneration (such as adipose tissue), repair (such as wounds, cartilage, bone, and nerves), *in vitro* bionic systems, clinical trials, and other disease research.

Key Words: Extracellular matrix; Decellularized adipose matrix; Decellularized adipose tissue; Adipose-derived extracellular matrix; Adipose tissue extracellular matrix; Adipose matrix; Stem cells; Soft tissue regeneration; Decellularization methods

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Core tip: Decellularized adipose matrix (DAM) is widely used in soft tissue regeneration because it has unique biological and physical properties and can provide a natural microenvironment for the growth and differentiation of stem cells. There have been many studies on DAM, and our objective is to comprehensively describe the preparation, characterization and application of DAM from the perspective of stem cells. We also describe the problems that still need to be solved in DAM research and possible future developments.

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INTRODUCTION

Soft tissue defects caused by trauma, tumors, and aging are often seen in clinical work, and tissue regeneration is undoubtedly one of the biggest challenges. Stem cell therapy has always played an important role in the field of regenerative medicine[1-3]. Stem cells achieve tissue metabolism and regeneration of post-traumatic defects through two unique attributes: (1) The ability to self-renew in the process of symmetric division; and (2) The ability to multidirectionally differentiate in the process of asymmetric division[4]. Although stem cells play an important role in soft tissue regeneration, risks and challenges also exist. Stem cells often require extensive expansion *in vitro*, which increases the risk of shortened telomeres, impaired function, and contamination[5]. It is common for stem cells to fail to stabilize in the recipient region after implantation, leading to a poor survival.

Therefore, from the application perspective of tissue regeneration, what stem cells need more is a natural biomaterial scaffold. It can provide stem cells with a microenvironment for growth and support for their colonization, adhesion, proliferation, and differentiation[6-10]. The dynamic and specific microenvironment of stem cell proliferation and differentiation is called a niche. The main component of the niche is the extracellular matrix (ECM), which can dynamically regulate the behavior of stem cells and provide extracellular clues for stem cell recognition[6,11]. The ECM is composed of various collagens, glycoproteins, and growth factors and seems to be a static network structure, but it is actually in a process of continuous remodeling with dynamic interaction with stem cells[12,13]. Generally, stem cell proliferation and differentiation are accompanied by changes in the ECM structure. For example, stem cells bind to matrix protein residues to change local conformation[14,15], or stem cell remodeling reveals hidden binding sites of the ECM to promote self-adhesion and proliferation[16,17].

Despite the advances in bionic technology and the rapid development of polymer materials science, there is still a huge challenge to fully simulate the biological properties of the ECM. Most artificial scaffolds fail to meet the requirements of biologically active vectors due to their lack of the ability to induce stem cell differentiation and the potential for dynamic interaction with cells[18-20]. Therefore, the acellular matrix of the target tissue/organ is an ideal bioactive scaffold. A cell-free, natural ECM scaffold can be obtained through a previously developed protocol. It is characterized by a rich biomolecular and unique three-dimensional (3D) structure that can play a key role even if the acellular matrix differs from the anatomical region of the donor site[21].

At present, there are many studies on the use of xenogeneic and allogeneic acellular matrix for different hosts[22,23]. The risk of immunogenic residues limits the application of xenogeneic tissues[24,25]. Human allogeneic tissues may be the most desirable source of the ECM. Adipose tissue comes from a wide variety of sources, and lipoaspirate is largely discarded every year as medical waste. With the rapid development of adipose tissue engineering, many researchers have tried to develop better acellular solutions to obtain decellularized adipose matrix (DAM)[26-28]. DAM continues to integrate with surrounding soft tissues and plays an important role in the entire regeneration process of the recipient area[29].

Currently, there are many protocols for the preparation of DAM. Different

preparation methods have different effects on key components of DAM, and further affect the growth of stem cells and regeneration of soft tissues[30-34]. This review outlines the importance of DAM to provide an inductive microenvironment for stem cells in tissue regeneration. In particular, considering the DAM for tissue engineering purposes, the different decellularization methods used are fully described (Figure 1). In addition, the problems that still need to be addressed with regard to DAM are also described, as well as possible future developments of these emerging bioscaffold materials.

LITERATURE SEARCH

A literature search was conducted using the PubMed Advanced Search Builder. An advanced search was performed using "decellularized adipose tissue OR adipose-derived matrix OR acellular adipose matrix OR decellularized adipose matrix" as the title elements, and identified 236 studies. After further analysis and evaluation on whether the title and abstract involve fat-derived ECM and whether the article is written in English, a total of 75 studies were included.

OVERVIEW OF ECM/DAM

The ECM is a 3D complex network structure composed of various collagens and glycosaminoglycans (GAGs), and provides effective biological information for the growth and differentiation of stem cells, and enables cell-cell and cell-ECM dynamic interaction through the establishment of a natural ecological microenvironment[35]. Stem cells continue to reshape the microenvironment created by the ECM, while the reshaped the ECM also constantly changes the behavior of stem cells[13]. This can keep the growth of stem cells in equilibrium with the degradation of the ECM and play a continuous and stable role in the entire tissue regeneration process[36]. At present, the ECM of various tissues including the skin[37], cartilage[38], bone[39], tendon[40,41], skeletal muscle[42,43], blood vessels[44,45], nerves[35,46], cornea[47], heart valves[48,49], myocardium[50,51], lung[52,53], liver[54,55], kidney[56,57], small intestine[58], and bladder[59] has been widely used in clinical or preclinical research in various fields.

In recent decades, the DAM extracted from a large amount of waste adipose tissue has aroused interest among researchers because of its abundant sources and excellent potential in soft tissue regeneration[60]. A large amount of adipose tissue can be obtained by using the developed method of degreasing and decellularization[26]. The DAM, which provides a natural microenvironment for the growth of stem cells [especially adipose-derived stem cells (ASCs)], has the following characteristics. First, the complex structure is composed of collagens I[22,61,62], IV[26,61,63,64], and VI[65], laminin[22,26,61,62,66,67], fibronectin[34,68], elastin[28], GAGs[22,28,62,63,69], and other biologically active macromolecules. Fibrillar collagen and glycoproteins provide structural stretch resistance and resilience[70], and play an important role in the entire dynamic remodeling process of stem cells. Second, the structure contains growth factors such as vascular endothelial growth factor (VEGF)[22,63,69,71], basic fibroblast growth factor (bFGF)[22,63,71], and transforming growth factor (TGF)-β[23], which are associated with specific ECM domains or proteins and play an irreplaceable role in the entire process of soft tissue regeneration[72,73].

In addition, there are different names about DAM, including decellularized adipose tissue[26,74-77], adipose-derived matrix[23,24], and acellular adipose matrix[78]. For the convenience of explanation, this article collectively names DAM from adipose tissue of different sources (including human, pig, mouse, *etc*.).

DIFFERENT PREPARATIONS OF DAM

There have been many studies on DAM (Table 1). Different preparation methods result in the retention or loss of DAM key components to varying degrees, and affect the growth of stem cells and regeneration status of soft tissues[65,79]. The goal of DAM preparation is to remove all immunogenic components (such as nucleic acids and fragments) from all cells, while retaining the biologically active components of the ECM (including collagens, proteins, growth factors, and GAGs) and suitable 3D structure and mechanical properties, to provide host stem cell growth and

Table 1 Different studies of decellularization and sterilization methods for preparation of DAM

Decellularization methods			Sterilization methods	Refs.
Physical treatments	**Chemical treatments**	**Biological treatments**		
Freeze–thaw, 3 cycles (-80 °C to 37 °C)	99.9% isopropanol	0.25% trypsin/0.1% EDTA 15000 U DNase, 12.5 mg RNase, 2000 U lipase	70% ethanol/1% penicillin and streptomycin/UV light/1% antibiotic/antimycotic	[26,30,74,75,83,84,96,101,102]
		0.05% trypsin-EDTA 100 U/mL benzonase	70% ethanol/1% penicillin and streptomycin	[63,66]
		0.05% trypsin 500 U/mL benzonase	0.1% peracetic acid in 4% ethanol	[61]
	99.9% isopropanol 1 mol/L NaCl	1 mmol/L EDTA + Lysis buffer (1% tergitol type NP-40, 0.1% SDS, 5 mmol/L EDTA, 0.4 mol/L NaCl, 50 mmol/L Tris-HCl pH 8, 1 mmol/L PMSF)	70% ethanol/1% penicillin and streptomycin	[65]
	100% isopropanol	0.25% trypsin-EDTA; 1 mL DNase + 1 mL RNase + 2 mL lipase	70% ethanol/1% penicillin and streptomycin	[135]
	0.5 mol/L NaCl/1 mol/L NaCl/isopropanol/Triton X-100	0.25% trypsinEDTA	1% penicillin and streptomycin	[69,71,93]
Freeze-thaw, 3 cycles (-80 °C to 37 °C) + ultrasonic	0.5% SDS + 100% ethanol	–	100% ethanol	[25]
Freeze-thaw, 35 cycles (-80 °C to 37 °C) + homogenization, 5 min (12000 r/min)	1% Triton X100 + 100% isopropanol + 1 mol/L NaCl	100 U/mL DNase 100 μg/mL RNase	–	[68]
Freezethaw, 4 cycles (-80 °C to 37 °C) + ultrasonic	96% ethanol 0.5% SDS	0.05% trypsin/0.05 mmol/L EDTA + DNase	–	[87]
Freezethaw, 5 cycles (-80 °C to 37 °C)	Isopropanol	0.25% trypsin/0.1% EDTA DNase I + RNase A	Ethylene oxide	[136]
Freeze-thaw, 5 cycles (liquid nitrogen to 37 °C)	99.9% isopropanol	0.05% trypsin-EDTA 20 ng/mL DNase I + 20 ng/mL RNase	1% penicillin and streptomycin	[67]
Freezethaw, 4 or 5 cycles (liquid nitrogen to Room temperature)	0.5 mol/L acetic acid	–	–	[88]
Freezethaw, 35 cycles (liquid nitrogen to Room temperature)	0.1% SDS	0.05% trypsin + 0.05% EDTA + 20 ng/mL DNase I + 20 ng/mL RNase	1% penicillin and streptomycin	[89]
Freezethaw, 618 cycles (liquid nitrogen to room temperature)	0.1% sodium azide + 1 mol/L NaCl + 4% sodium deoxycholate	2000 K units DNase	–	[78]
	1% Triton X-100	2000 K units DNase		
Homogenization, 5 min (12000 rpm)	1 mol/L NaCl/0.5% SDS	0.2% DNase + 200 μg/mL RNase	–	[27,28,81]
	–	–	–	[104,137]
	0.5% SDS + 100% isopropanol	–	–	[138]

Homogenization, 5 min + ultrasonic	–	0.25% Pancreatin	Ethylene oxide	[79]
Homogenization, 3 min (12000 r/min)	SDS	–	–	[90]
	4 mol/L urea	4 mol/L Gu	Ethylene oxide	[100,139]
Homogenization (twice)	2 mol/L urea+70% ethanol	2 U/mL dispase II + 4 mol/L GuHCl	Dialysis against chloroform	[23,24]
Homogenization	2 mol/L urea buffer	–	70% ethanol/1% antibiotic/antimycotic solution	[65]
Constant stirring	1% SDS or 2.5 mmol/L sodium deoxycholate	2.5 mmol/L sodium deoxycholate + 500 U porcine lipase + 500 U porcine colipase	365 nm UV light	[62,85]
Constant stirring	1% SDS	2.5 mmol/L sodium deoxycholate + 100 μg/mL lipase + 50 ng/mL colipase; 50 μg/mL DNase + 50 μg/mL RNase	Ethylene oxide	[86]
Mechanical processing	0.1%, 1%, 3%, or 5% Peracetic acid + 1% Triton X-100	600 U DNase	–	[31,94]
	3% Triton X100 + 4% sodium deoxycholate + 4% ethanol/0.1% peracetic acid + 100% n-propanol	0.02% trypsin + 0.05% EDTA	4% ethanol + 0.1% peracetic acid	[22]
$SCCO_2$ (180 bar)	Ethanol	–	SC-CO_2	[91]
–	1% sodium dodecylsulfate + 100% isopropanol	2.5 mmol/L sodium deoxycholate + 500 U lipase + 500 U colipase	5000 IU penicillin and 5 mg/mL streptomycin	[32]
	0.5% SDS + isopropanol + 0.1% peracetic acid + 4% ethanol	–	0.1% peracetic acid+4% ethanol	[92,103]
	1% Triton X-100	10, 20 and 100 IU/mL DNase I	–	[82]
	1-propanol	Sodium deoxycholate	Peracetic acid	[33]
	Organic solvent + surfactant/ethanol-based solution	–	Peracetic acid	[34,64]

SDS: Sodium dodecyl sulfate; EDTA: Ethylenediaminetetraacetic acid; SC-CO2: Supercritical carbon dioxide; Gu: guanidine.

differentiation microenvironment after transplantation[80]. However, all current decellularization schemes will inevitably cause different degrees of damage to the structure and composition of DAM[60].

The current effective decellularization protocol is achieved by a combination of physical, chemical, and enzymatic methods (Table 2). Usually, the first step is to destroy the cell membrane components by physical means (freezethaw cycle[26] and homogenization[28,81]). Second, chemical methods include the use of detergents[33] /nondetergents[82] to dissolve cytoplasmic and nuclear components and alcohols (such as isopropanol[26,83,84]) to remove lipid residues. Finally, cell residues and degraded nucleic acid fragments are removed by enzymatic methods[28,68,81] (including DNase and RNase). The above steps can be combined with continuous mechanical stirring and shaking, to shorten the action time of reagents, improve efficiency, and reduce structural damage[62,85,86]. In addition, in order to avoid the immune response caused by

Table 2 Comparison of each physical, chemical, and biological treatments in the adipose tissue decellularization protocols

Agent/method	Function or advantages	Impact or disadvantages
Physical		
Freezing thawing	Ice crystals destroy cell membranes	Ice crystals also destroy the continuity of
	Preserve component integrity	DAM composition and microstructure
	Reduce immune response	
Homogenization	Fully destroy the cell membrane structure and promote dissociation from basement membrane	Mechanical shear forces break the microstructure and component continuity
Constant stirring	Cleave the cell membrane	Stirring forces destroy microstructure
	Full exposure accelerates the effect of chemical agents	Mechanical properties are affected
Mechanical processing	Promote cell membrane rupture and release from the basement membrane	Pressure directly destroys microstructure; ultrastructure and basement membrane integrity are destroyed
SC-CO_2 treatment	Supercritical inert gas penetrates tissues to remove cell residues/sterilization	Entrainer may reduce structural composition; supercritical pressure may destroy the structure
Ultrasonic	Ultrasonically break cell membrane	-
Chemical		
Hypotonic/hypertonic solutions	Dissociate DNA from proteins; Osmotic pressure ruptures cell membranes	Little influence on the structure and composition of DAM
Alcohols		
Isopropanol	Cell dehydration, cell membrane lysis	DAM protein components are precipitated; destruction of ultrastructure; degreasing alone has poor effect
Ethanol	Effectively remove lipid residue	
Acids and bases		
Acetic acid	Hydrolyze biomolecules to remove residual nucleic acids; little effect on the structure; better retention of GAGs components	Some collagen components are destroyed and removed; reduced strength of DAM; collagen, growth factors, and GAGs are damaged
Peracetic acid		Little effect on the structure and composition of DAM
Nonionic detergents		
Triton X-100	Disturbing DNA–protein, lipid–lipid, and lipid–protein associations; moderate effect/stable in solution	Destruction of ultrastructure; remove GAGs
Agent/ Methods	Function or advantage	Impact or disadvantage
Ionic detergents		
SDS	Effectively remove cellular nucleic acid components/destruction of cell membrane phospholipids and lipoproteins/dissolving antigen and eliminating immune complexes	Disturbing protein–protein association; growth factor removal; destroy ultrastructure, GAGs ingredients; residue of the reagent causes cytotoxicity
Sodium deoxycholate		
Triton X-200		
Biologics		
Trypsin	Cleavage of the C-side peptide bond of Arg and Lys	Remove fibronectin, elastin, and GAGs components; damage degree of DAM composition and microstructure is highly time-dependent
Nucleases (DNase, RNase)	Cleavage nucleotides sequence	Difficult to remove residue from DAM; residual effects on host recellarization; causes host immune response
Lipase and colipase	Remove residual lipids	Destruction of ultrastructure; removes GAGs; efficiency of lipid removal is low
EDTA	Dissociation of metal ions plays a supporting role in tissue decellularization	Destruction of protein–protein linkages; poor application alone

SDS: Sodium dodecyl sulfate; EDTA: Ethylenediaminetetraacetic acid; SC-CO_2: Supercritical carbon dioxide.

the residues of chemical and enzymatic substances, thorough washing at each step is essential[26]. Flynn *et al*[26] was the first to prepare complete DAM through the above-mentioned comprehensive method. After 5 d of nondetergent solution, DAM was finally obtained with a high retention rate (30%-45% of the original amount). After a series of characterizations, components such as collagens I and IV and laminin of DAM, which are important for adipogenesis and stem cell proliferation, are retained[26]. This method has been widely used and improved by many researchers subsequently.

Physical treatment

The physical treatment method has had the following improvements. First, the number of cycles is increased based on three freezethaw cycles[67,68,78,87-89] or the freezing temperature is reduced from -80 to -196 °C (liquid nitrogen)[67,78,88,89] or adding ultrasonic treatment[25,87] during the freeze-thaw process. According to the research, within a certain range (1-5 times), increasing the number of freezethaw cycles will not have much effect on the microstructure of DAM, and cell debris and residues cannot be removed only by freezethaw treatment. With the increase in the number of freeze-thaw cycles (6-18 times), the microstructure of DAM is damaged[78]. Second, Choi *et al*[28,81] changed the freezethaw treatment to homogenization. The homogenization can quickly and fully damage the cell membrane structure, but the longterm effect of mechanical shear force destroys the microstructure of DAM and results in the loss of specific components (such as laminin)[28,81]. Subsequently, Kim *et al*[90] reduced the time of homogenization, to protect the integrity of DAM structure and composition[23,24]. Third, Young *et al*[62] replaced freezethaw and homogenization with continuous stirring or mechanical pressing, with the aim of accelerating chemical and enzymatic surface contact in the later stages to shorten reaction time[31,86]. Fourth, Wang *et al*[91] tried to use the advanced technology of supercritical carbon dioxide (SC-CO_2), and only used ethanol as an entrainer to decellularize and degrease adipose tissue to obtain DAM. Finally, Pati *et al*[92] abandoned the physical processing steps and directly used chemical and enzymatic methods to obtain DAM[33,34,64].

Chemical treatment

Chemical methods also have different application modifications. Alcohol, acid/base, or ionic/nonionic detergents affect the structure and composition of DAM to varying degrees. Hypertonic saline dissociates DNA from proteins in a gentle way to achieve decellularization and has little effect on the microstructure and composition of DAM[69,71,89,93]. Although the types and concentrations of alcohols are not the same, there seems to be no significant difference in lipid removal[25]. Alcohols such as isopropanol, *n*-propanol, and ethanol are superior to lipase in removing lipids from tissues. They can remove lipids in a short period of time, but at the same time, they can also denature the protein components of DAM (such as collagen and LN) and destroy the ultrastructure[22]. Therefore, caution should be exercised when using them. Acidic reagents can hydrolyze the biomolecules of tissues, acetic acid may cause damage to certain collagen components and reduce the structural stability of DAM[88], while peracetic acid is a commonly used disinfectant and can also be used as a decellularizing agent because it can gently remove residual nucleic acids. It has little effect on the composition and structure of DAM[22,31,92,94]. In general, ionic detergents (including SDS and sodium deoxycholate) are more effective in removing cellular components than nonionic detergents (such as Triton X-100), but they also damage the ultrastructure of DAM, and more GAGs and growth factor components are also removed[95]. The comprehensive application of multiple chemical agents may aggravate the loss of DAM components (such as GAGs and collagen) and destruction of the structure (including mechanical properties)[60].

Biological treatment

Nuclease, trypsin, lipase, and EDTA are widely used as biological reagents. The removal of cell debris and residual lipids or degradation of nucleic acid fragments is their main functions. It is also difficult to use enzymes alone to completely remove cell residues. In addition, the residues of enzyme reagents may further affect the growth and differentiation of stem cells, and even cause an adverse immune response in the host. Nucleases (DNase, RNase, *etc*.) cleave nucleotide sequences after cell membrane rupture and help remove nucleic acid residues[83,84,96-99]. Trypsin and a chelator (such as EDTA) are often used in combination. Trypsin can efficiently remove cell residues and destroy collagen, elastin, GAGs, and other components, and the damage to the structure and components of DAM also increases with the time of action (time

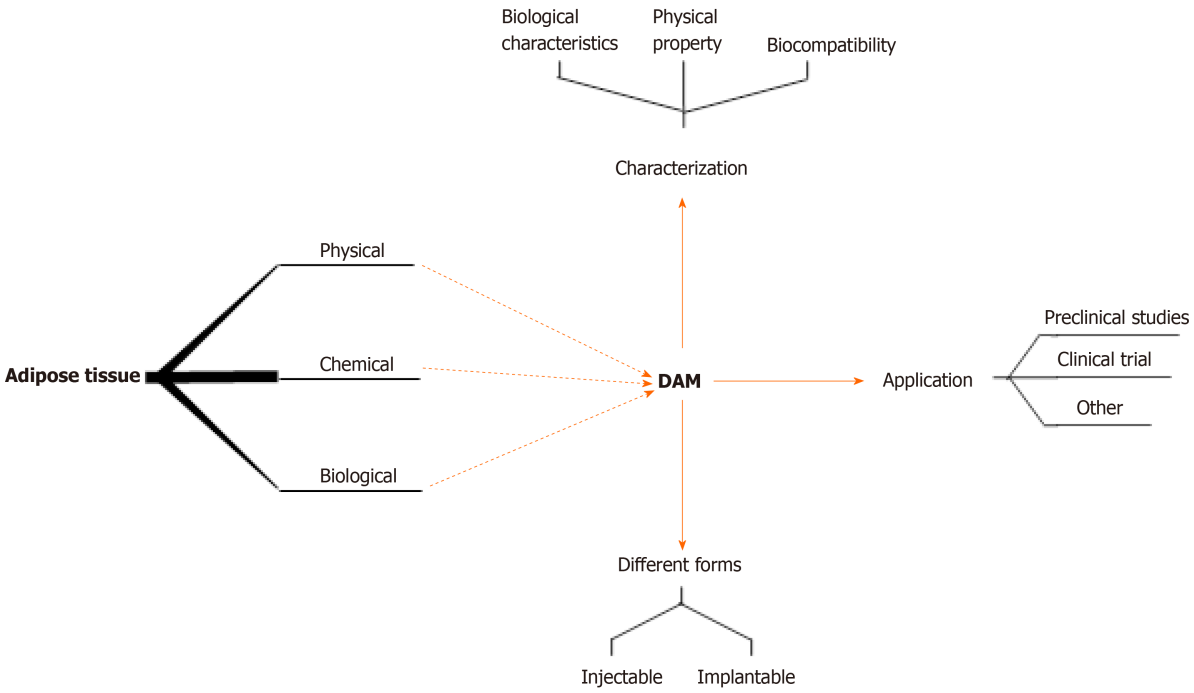

Figure 1 Preparation, characterization, different forms, and applications of decellularized adipose matrix. DAM: Decellularized adipose matrix.

dependent)[83,84,93,96-99]; EDTA helps DAM proteins dissociate from cells. These two reagents have a poor effect when used alone, and only when combined can they play a synergistic role, and EDTA can reduce trypsin digestion time and reduce tissue damage[22,67,89]. Lipase and co-lipase are often used in combination to remove residual lipids[32,62,85]. In addition, Poon *et al*[23] used guanidine alone or combined with hydrochloric acid to remove lipid residues, and the growth factors detected in DAM were well retained[24,100].

Different sterilizations of DAM

After preliminary preparation, it is important to sterilize the prepared DAM when conducting *in vivo* or *in vitro* experiments. This mainly removes bacteria and viruses. At present, the methods for sterilizing biological scaffolds mainly include alcohols, acids, ethylene oxide, UV irradiation, and SC-CO_2. The prepared DAM is usually stored in a 1% penicillin and streptomycin solution at 4°C[96-98,101,102], and then sterilized with 70% ethanol solution. Some researchers use 100% ethanol alone to sterilize biological scaffolds[25]. Four percent ethanol solution and 0.1% peracetic acid are often used in combination for sterilization, with significant effect[61,92,103], and they also have little effect on the structure and composition of DAM. Wang *et al*[79] used ethylene oxide for sterilization, but the effect on the microstructure of DAM is unclear. However, there is no doubt that residual reagents after ethylene oxide treatment may cause adverse host reactions and affect the function of the biological scaffold after implantation. In addition, Young *et al*[62,85] used UV radiation for sterilization. During the sterilization process, the collagen component of DAM may be partially denatured, which may accelerate degradation of the stent material in the body[62,85]. More research is needed. As an innovative method, SC-CO_2 is applied to the decellularization of adipose tissue, and it sterilizes biological materials[91]. The specific impact on biological materials needs further comparative research.

CHARACTERIZATION OF DAM

Just as researchers have developed different preparation schemes, there is currently no uniform standard procedure for characterizing DAM materials. It is impractical to remove all cellular residues, but quantitative analysis of residual cellular components (such as phospholipids and double-stranded DNA) is possible. At present,

characterization of DAM generally includes: Simple evaluation of the general effects of decellularization and degreasing of materials using simple histological staining and electron microscopy (EM); and DNA quantification, biochemical analysis, and mechanical stress testing to further evaluate various aspects of DAM. This section provides a brief summary.

DAM biological characteristics test

For detection of cell residues, the first approach is histological staining and biochemical analysis. Simple histological staining including hematoxylin and eosin and oil red O staining to roughly check whether the nuclear and lipid components are removed[26,28,31,62,104]. Immunohistochemical staining includes DAPI and Hoechst staining to determine the presence of visible nucleic acid and cellular component residues. This is followed by further biochemical tests, including DNA quantification and reverse transcription-polymerase chain reaction analysis. Gilbert *et al*[105] have suggested the criteria for acellular matrix: DNA content < 50 ng/mg dry weight double-stranded DNA and DNA fragment length < 200 bp. This standard may be one of the most important for the application of biological materials, because hindering the further growth and differentiation of stem cells and causing adverse host reactions may be directly related to DNA residues.

DAM structure and physical property detection

In terms of detecting the structure and composition of DAM, the microstructure and structural stability of DAM are first detected by scanning electron microscopy (SEM) and mechanical stress testing[26,92]. As mentioned above, the effect of microstructure on stem cells may be crucial, where stiffness is a key indicator[106]. The process of stem cells responding to their environment after sensing external forces is called mechanical transduction. All types of stem cells have the ability to sense the structure and stiffness of DAM[11]. Cell morphology, skeleton, and migration can interact with DAM in the short term. The more important effects of proliferation and differentiation are long-term[106]. The porosity and 3D microstructure of DAM were observed by SEM[26]. Mechanical stress tests include Young modulus, storage modulus, and loss modulus, which are used to comprehensively evaluate the mechanical integrity, elasticity and rheological properties of materials[103]. The compression mechanical test of DAM was carried out with a universal testing machine. The sample was compressed to 50% of the initial height at a low constant rate. The compressive modulus was calculated using a linear region of stress-strain curve[103]. Perea-Gil *et al*[107] used the atomic force microscopy to determine the mechanical properties of decellularized myocardium tissue samples, such as stiffness and Young's modulus. This is followed by further analysis of its composition by staining and biochemical analysis. Masson trichrome staining can quickly and easily detect gross collagen components. Immuno-histochemical staining can detect components such as types I, IV, and VI collagen, laminin, fibronectin, and elastin in more detail[26,62]. However, there is currently no effective detection for the quality of these proteins in DAM. The specific contents of DAM (such as TGF-β and VEGF) and GAGs can be accurately detected and analyzed by ELISA[33].

DAM biocompatibility testing

In terms of biocompatibility, coculture of DAM with mesenchymal stem cells (mainly ASCs) to detect the adhesion and proliferation of stem cells on the material is required[30,69,82]. Flynn *et al*[26] verified the fat regeneration potential of the acellular matrix by detecting expression of adipogenic genes such as *PPARγ* and *C/EBPa*. They also found that the GAPDH activity of DAM differed when prepared from adipose tissue with different body mass index (BMI; BMI is inversely proportional to GAPDH activity)[26]. LIVE/DEAD analysis was performed by staining living and dead cells using a combination of Calcein and EthD-1[28,62]; Kokai *et al*[33] used calcein AM to further stain the cocultured material, and then used laser confocal imaging to show different colors to infer the depth of the stem cell infiltration of the scaffold material. SEM at different times shows the dynamic interaction process of stem cells and materials at the microscopic level. At the same time, the authors exposed ASCs to the adipose matrix for 21 d, and then used boron-dipyrromethene staining, followed by confocal imaging to observe the increase in lipid content. After transplanting DAM with/without stem cells into the subcutaneous tissue of animals, hematoxylin and eosin, Masson, and perilipin A immunofluorescence staining were used to observe adipose tissue regeneration *in vivo*[33].

DIFFERENT FORMS OF DAM

After degreasing and decellularizing, DAM can be processed into different shapes of biological scaffolds and used with or without stem cells. It can be roughly divided into injectable and implantable types according to different usage methods.

The main advantages of injectable DAM are convenience and noninvasiveness, including powders and gels. DAM powder is digested into gel with pepsin, and then the pH is adjusted to the normal range with sodium hydroxide solution. During use and storage, the temperature should be controlled below 10 °C to prevent curing[62]. DAM (powder or gel) is usually absorbed to varying degrees after implantation. Some researchers have tried to use polymer crosslinking, which slows down the rate of stent degradation and enhances angiogenesis and fat induction[86].

The advantage of implantable DAM is that the structural integrity is preserved, including foam and sheets. Foam-like DAM is lyophilized into porous foam by dissolving with α-amylase, which has a milder effect than pepsin. Another type of bead foam is that the DAM solution is rapidly frozen after electrospray technology, and then freeze-dried at low temperature. Chemical crosslinking is avoided, and *in vivo* experiments have confirmed that foamed DAM has fat-forming ability and biocompatibility[83]. The DAM is cast in a superficial mold, and a sheet-like DAM is obtained after freeze-drying. Experiments have shown good mechanical integrity and multicellular compatibility[93].

DAM can also be combined with other artificial composite materials, such as methylcellulose (MC)[100], methacrylated glycol chitosan (MGC)[84], methacrylate chondroitin sulfate (MCS)[84], and polycaprolactone (PCL)[92], to be used as stem cell growth scaffolds. It has been shown *in vitro* that composites can effectively enhance host stem cell invasion and angiogenesis[32]. The use of PCL/DAM composites as bio-ink for 3D printing has boomed in recent years. This open porous structured scaffold has been verified *in vitro* to have better oxygen and nutrient exchange capacity than ordinary DAM gels[92,103].

PRECLINICAL STUDIES ON APPLICATIONS OF DAM

At present, as a biological scaffold for tissue engineering, DAM is used alone[33] or in combination with stem cells[69] in various fields, including adipose tissue engineering, wound healing, nerve repair, cartilage and bone tissue engineering, and *in vitro* biomimetic system research.

Adipose tissue engineering

DAM is the most widely studied as a filler for soft tissue defects. Stem cells are seeded on DAM and injected or transplanted into subcutaneous tissue, which provides a natural microenvironment for the growth of stem cells to further promote adipogenesis and angiogenesis. After coculture of DAM and ASCs, DAM can express the adipogenesis markers PPARγ and C/EBPα (major regulators of adipogenesis and differentiation) at high levels without exogenous adipogenesis induction compared to ordinary monolayer cultures such as Triplicate tissue culture polystyrene and Cell Aggregate[26]. Expression of these two genes plays a cross-regulatory role in the entire adipogenic differentiation and plays an important role in maintaining the transformation of adipocytes to mature phenotypes. After ASCs/stromal cells were seeded in DAM microcarriers and then cultured in a low-shear fine-tuning culture system for adipogenic culture, expression of the adipogenic genes *PPARγ*, *C/EBPa*, and *LPL* was higher than that of ordinary gelatin microcarriers[30]. This indicates that DAM plays an important role in mediating adipogenic differentiation of ASCs. After implanting DAM loaded with ASCs into the subcutaneous tissue of rats or nude mice[69], the implanted area showed significant recellularization and angiogenesis[32,108]. This shows that DAM plays an important role in supporting stem cell infiltration and tissue remodeling. Han *et al*[98] used ASCs for seeding on DAM bioscaffolds, and then implanted them into the subcutaneous tissue of rats. Cell tracking technology was used to verify that the new adipose tissue originated from the host[98], and ASC-seeded DAM contributed to fat formation by promoting neovascularization and modulating the inflammatory response. In addition, research on the combination of DAM and artificial composites is also developing. For example, light crosslinked MGC/MCS and DAM form a composite biological scaffold. *In vitro* studies showed that DAM can also enhance the viability, retention, and lipid accumulation of ASCs. MCS composites containing 5 wt% DAM were transplanted into the subcutaneous tissue of rats. After

12 wk, it was observed that DAM seeded with ASCs significantly increased regeneration of adipocytes[83,84]. ASCs were seeded in 3D printed PCL/DAM composite bioscaffolds and then implanted into the subcutaneous tissue of nude mice. The results after 12 wk showed that there were a reasonable number of mature adipocytes and functional blood vessels in the DAM area[92].

Wound healing

Clinically, deep burns or large skin trauma are usually treated by flap transfer surgery. Patients often have infections, fluid loss, and electrolyte disorders[109-112]. Lee *et al*[113] used DAM sheet scaffold dressing to treat full-thickness skin wounds on the back of rats. The results showed that the wound healing rate, epithelial formation rate, and microvascular density were significantly higher than those of ordinary wound dressings[113]. Woo *et al*[114] applied a double-layer dressing (the upper layer was made of titanium dioxide and chitosan film by electrospinning, and the lower layer was DAM) to a full-thickness wound in rats. It was showed that it can accelerate the induction of fresh granulation tissue regeneration and reduce epidermal scar formation. These results indicate that the components of DAM (such as collagen, laminin, fibronectin, and GAGs) and various growth factors (such as VEGF and bFGF) can promote regeneration of the ECM in the wound area, further recruit adipose stem cells, fibroblasts, and epithelial cells to accelerate tissue reconstruction and vascular regeneration[114].

Nerve repair

Regeneration is difficult after nerve tissue damage. Lin *et al*[89] used DAM containing ASCs in a rat cavernous nerve injury model, and showed the best recovery of erectile function in rats with DAM seeded with stem cells, but the results did not reach statistical significance due to large differences. However, we also saw substantial recovery of erectile function and histological improvement associated with DAM seeded with ASCs, which has potential for clinical application in the future[89].

Cartilage and bone tissue engineering

Cartilage is difficult to repair due to its nonvascular nature and long-term wear and tear. Cartilage-derived ECM has been used in research on cartilage regeneration[115,116]. The cartilage decellularized matrix seeded with ASCs can completely repair articular cartilage defects with hyaline cartilage. At the same time, the contents of GAGs and type II collagen and biomechanical properties have been proven to be comparable to those of natural cartilage[115]. Adipose-derived mesenchymal stem cells are also used for cartilage regeneration, which differentiates into chondrocytes and can produce important proteins required for articular cartilage (such as the mucus glycoprotein Lubricin)[117-119]. This alternative treatment has proven to be effective. However, due to limited resources, the prospect of clinical application is limited. Choi *et al*[81] have prepared an ECM/stem cell composite, which formed cartilage-like tissue after being cultured in cartilage induction medium for 45 d, and at the same time, the expression of cartilage-specific GAGs and type II collagen increased. This shows that DAM containing endogenous active factors can support cartilage differentiation of human ASCs and help with cartilage-specific glycoprotein and collagen synthesis[81], which has potential clinical value in the synthesis of cartilage-like tissue.

Bone has significant capacity of regeneration, but patients with large-scale bone defects need surgical autogenous bone transplantation, which causes damage and infection of the donor site[120-122]. Artificial composite scaffolds are poorly biocompatible and cannot support vascular regeneration and bone tissue growth[122], while the source of acellular bone tissue is insufficient to meet clinical needs[123,124]. Mohiuddin *et al*[125] used DAM hydrogels to treat C57BL/6 mice for critical size repair of femoral defects. The results showed that hydrogel can enhance expression of type I collagen and osteopontin, while the hydrogel-treated group significantly enhanced bone regeneration *in vivo*[125].

Bionic research in vitro

The composition and structure of DAM show that it can mimic the natural microenvironment of stem cells and even tumor cells in the body. Research shows that when seeding on DAM or chemically modified DAM[30,31,33,64,74,84,92,96,126], ASC[76,81,84,89,93,85,82,98,104,127], smooth muscle cells[90], umbilical vein endothelial cells[90], chondrocytes[90], and neuroblasts[25] can maintain high viability and excellent proliferation, indicating that DAM may become the ideal 3D culture system for the large-scale expansion of stem cells *in vitro*. Dunne *et al*[93] used DAM as a 3D cell

culture system and established a human breast cancer *in vitro* bionic system to study the growth of breast cancer cell lines (MCF-7, BT474, and SKBR3) and the sensitivity of anticancer drugs (lapatinib and doxorubicin). This restored the original characteristics of breast cancer cell growth *in vivo*, and expression of adhesion molecules in tumors *in vivo*. This is undoubtedly beneficial to the screening and research of antitumor drugs[93].

CLINICAL TRIAL ON APPLICATION OF DAM

Most studies on DAM were preclinical studies combined with stem cells. Recent research has shown that stem-cell-free DAM can also promote adipose tissue regeneration. For the first time, Kokai *et al*[33] applied DAM alone to a clinical trial. DAM prepared from cadaveric human adipose tissue was applied to the subcutaneous tissue of nude mice and the subcutaneous wrist dorsum of humans. After 24 wk *in vivo*, the material retention rate was 44% ± 16%, and the regeneration of adipocytes could be clearly observed by immunofluorescence assays, such as perilipin A. Clinical trials evaluated biocompatibility, volume retention, and soft tissue regeneration over a 16-wk period. There were wrist pain, redness, swelling, and itching at the initial stage during the observation period, which may have been related to the initial inflammation. At 16 wk, the average graft retention was about 47%. No inflammation or necrosis was observed in pathological observation, and adipose tissue was formed around the dilated vessels. Although the study had many limitations, the role of DAM in promoting adipose regeneration was verified[33].

OTHER APPLICATIONS

As an important endocrine organ, adipose tissue is closely related to many metabolic diseases such as diabetes mellitus (DM). The specific mechanism of how the ECM is involved in regulating adipocyte metabolism is unclear. The relationship between DAM and DM has been the focus of research. The ECM of adipose tissue is closely related to metabolic diseases. Factors such as hypoxia, inflammation, and fibrosis of the ECM are related to insulin resistance and DM[128-132]. After collecting visceral and subcutaneous adipose tissue, Baker *et al*[99] used metabolic assays to measure glucose uptake, lipolysis, and adipogenesis in adipocytes in normal cell culture and 3D DAM culture. The results show that DAM with diabetes can cause metabolic dysfunction in adipocytes of non-DM patients; nondiabetic DAM can rescue metabolic dysfunction in adipocytes of DM patients. This indicates that the ECM is involved in regulating glucose uptake and lipolysis as a target for manipulating adipose tissue metabolism[99].

Autologous fat transplantation is used in the clinic to treat vocal cord paralysis. Although biocompatible, its unpredictable absorption rate is also a limitation[133,134]. Kim *et al*[100] used the MC/DAM composite hydrogel for rabbit vocal cord paralysis studies, and showed that the composite hydrogel group had no early absorption after 8 wk, and the physiological symmetry of vocal cord vibration returned to normal levels. The composite hydrogel overcomes the shortcoming of the indefinite absorption rate of autologous fat transplantation. Its good biocompatibility and positive functional recovery make it possible for clinical use as stable vocal cord enhancement laryngoplasty[100].

CONCLUSION

In the past 10 years, the preparation of DAM has been improved by different decellularized techniques. The material retains the main collagen components and most structural proteins and growth factors. This biologically active system can recruit host stem cells and mimic the growth microenvironment to promote the regeneration of soft tissues. Furthermore, DAM can be processed into different forms for different applications. For DAM, preliminary progress has been made in soft tissue regeneration and metabolic diseases. The combination of DAM with stem cells or growth factors has important value in preclinical studies such as wound healing, nerve repair, cartilage and bone tissue engineering, and bionic system. It is believed that with further exploration and research on DAM, it will play a major role in the field of stem cells and soft tissue regeneration.

However, residues of chemical and enzymatic reagents in the current preparation methods are still problems to be resolved. At the same time, the microstructural destruction and component loss (such as collagen and protein) caused by decellularized reagents and inefficient acellular technology are problems that require improvement. Will it be possible to develop a high-efficiency and high-retention decellularization technology based on physical methods to obtain a more complete DAM in the future? Considering this, a deep understanding of the cascade interaction in tissue regeneration, which is induced by DAM structural proteins and infiltrating host stem cells, is required.

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MINIREVIEWS

Vitamin D and calcium signaling in epidermal stem cells and their regeneration

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Abstract

Epidermal stem cells (SCs) residing in the skin play an essential role for epidermal regeneration during cutaneous wound healing. Upon injury, distinct epidermal SCs residing in the interfollicular epidermis and/or hair follicles are activated to proliferate. Subsequently, SCs and progeny migrate, differentiate and restore the epidermis. We review a role of the vitamin D signaling through its receptor of vitamin D receptor (*Vdr*) in these processes. *Vdr* conditional knockout (cKO) mouse skin experiences a delay in wound re-epithelialization under low dietary calcium conditions, stimulating our efforts to examine a cooperative role of *Vdr* with calcium signaling through the calcium sensing receptor in the epidermis. We review the role of vitamin D and calcium signaling in different processes essential for injury induced epidermal regeneration during cutaneous wound repair. First, we discuss their roles in self-renewal of epidermal SCs through β-catenin signaling. Then, we describe epidermal remodeling, in which SCs and progeny migrate and differentiate to restore the epidermis, events controlled by the E-cadherin mediated adherens junction signaling. Finally, we discuss the potential mechanisms for vitamin D and calcium signaling to regulate injury induced epidermal regeneration mutually and interdependently.

Key Words: Vitamin D; Calcium; Stem cells; Epidermis; Regeneration; Wound healing

Core tip: Vitamin D and calcium signaling play critical roles in epidermal stem cells and progeny to regenerate the epidermis during cutaneous wound healing. Their regulation of these processes is mediated at least in part through β-catenin and E-cadherin mediated

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INTRODUCTION

Chronic skin wounds are estimated to affect 6.5 million patients in the United States at a cost of over $25 billion[1]. A disproportionate number of these wounds are found in patients suffering from a variety of medical conditions including poor nutrition. A survey of patients with chronic leg ulcers by one of our clinical collaborators, Dr. Gasper, found vitamin D deficiency (25OHD levels less than 20 ng/mL) and decreased serum calcium (below 8.7 mg/dL) in nearly 50% of these patients (unpublished observation with permission). Moreover, chronic kidney disease but without diabetes mellitus or cardiovascular disease led to delayed healing of abdominal surgical wounds correlating inversely with 25OHD levels[2,3].

Adult stem cells (SC) residing in the skin play an important role in the regeneration of the epidermis after wound injury. Understanding the mechanisms regulating these adult SC is central for understanding epidermal regeneration during cutaneous wound healing. Skin epithelia are derived from the ectoderm and differentiate into the interfollicular epidermis (IFE), sebaceous gland (SG) and hair follicle (HF) during the embryonic developmental process. After birth, adult SC residing in the basal layer of the epidermis (eSC), isthmus (iSC) and bulge (bSC) regions of the HF regenerate the IFE, SG and HF, respectively[4-7]. In the epidermis, eSCs produce transient amplifying cells, which leave the basal layer and produce differentiation marker proteins such as involucrin (IVL), keratin 1 (KRT1), loricrin (LOR) and filaggrin (FLG) in a sequential process. In the proximal portion of the HF, iSCs maintain the SG. The bSCs regenerate HFs in a cyclic manner through activation signals initiated by the dermal papilla adjacent to the bSC.

However, when the skin is injured, these stem cells and progeny from all regions of the HF, SG and IFE contribute to regeneration of the epidermis at least initially[4,7] but to a different extent. Ito *et al*[8] found that bSCs provide around 25% of the newly regenerated epidermal cells by using an inducible *Krt*15-crePR/R26R transgene, although these cells did not persist during the healing process. Levy *et al*[9] demonstrated that the SCs from HF infundibulum also contribute to re-epithelialization by using a Shh Cre/R26R transgene, and these cells remained in the newly formed epidermis. However, eSC in the IFE make the greatest and most persistent contribution to epidermal regeneration[4]. Moreover, Langton *et al*[10] showed that bSC are not required for wound re-epithelialization because epidermis lacking HF as in the paw also re-epithelize the wound but with a delayed rate. The self-renewal of these SCs and their injury induced activation as well as migration and differentiation of progeny are controlled by various signal pathways.

The vitamin D receptor (VDR) is enriched in these stem cells. Its ligand,1,25$(OH)_2D_3$, a well-known regulator of epidermal differentiation and proliferation[11] can be produced in these epidermal cells. The epidermis is the major source of vitamin D for the body. Vitamin D is produced from 7-dehydrocholesterol by irradiation with UVB from the sun. As noted keratinocytes in the epidermis metabolize vitamin D to its active ligand 1,25$(OH)_2D_3$[11]. Tian *et al*[12] observed that topical 1,25$(OH)_2D_3$ enhanced wound healing. Vitamin D signaling regulates epidermal stem cells by interaction with other signaling molecules during cutaneous wound healing.

β-catenin signaling plays an important role for the maintenance of adult SCs in skin in both the HF and IEF. The role of β-catenin in bSC function has been extensively reported[13,14], but less attention has been paid to its role in eSC function. However, a sensitive probe for β-catenin signaling in the IFE is that of an *Axin 2* Cre reporter that demonstrates a role for β-catenin in eSC activation[5,13]. VDR appears to support activation of adult SCs through interaction with β-catenin. VDR binding to β-catenin promotes VDR transcriptional activity and facilitates cell fate determination[15,16]. In the HF, VDR is essential for β-catenin signaling and bSC activation[17,18]. VDR binding to β-catenin in the AF2 domain supports transcription for the bSC marker *Krt15* and HF

differentiation genes such as *PADI3* and *S100a3*[16]. Our observations also suggest that the same may be true for eSC in the epidermis as keratinocytes lacking *Vdr* show a blunted wound induced activation of β-catenin target gene expression at the wound edge as described below.

Calcium signaling also is important for cutaneous wound repair through interaction with vitamin D signaling. VDR profoundly affects calcium signaling within the cell including the induction of the calcium sensing receptor (CaSR) and a number of the pathways regulated by both calcium and vitamin D that are involved in epidermal differentiation. CaSR is a seven transmembrane domain, G protein coupled receptor first identified in parathyroid cells[19], that we cloned from keratinocytes[20]. CaSR is essential for the keratinocyte response to calcium[21,22], but like the VDR its role in wound healing has received little attention. The *in vivo* role of CaSR in calcium signaling in epidermis is demonstrated by a *Casr* null mice, in which the transmembrane domain and intracellular *Casr* is deleted from *Krt14* expressing keratinocytes[23]. The expression of the CaSR is increased by $1,25(OH)_2D_3$, causing the keratinocyte to be more sensitive to calcium actions[24,25]. Moreover, to our surprise, CaSR deletion reduces VDR expression[23].

Calcium signaling mediates epidermal remodeling after wound injury through adherens junction (AJ) signaling. In skin epithelia, the core molecular components of the AJ are cadherins and their binding partners β-catenin, α-catenin, and p120-catenin. The AJ signaling plays an essential role in epidermal differentiation *via* its role in activating phospholipase (PLC)γ, that in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG), signaling molecules critical for the differentiation process through their release of calcium from intracellular stores (IP_3) and activation of several protein kinase Cs[26]. Formation of the E-cadherin/catenin complex is regulated by both VDR and calcium[26]. The E-cadherin/catenin complex also links to the underlying cytoskeleton *via* α-catenin that helps form an epithelial sheet in enabling the cell migration required to re-epithelialize the wound in addition to promoting its subsequent differentiation to regenerate the epidermis[27].

In this review, we focus on the roles of vitamin D signaling and calcium through their receptors (VDR, CaSR) in the epidermis for the control of adult SCs and progeny during the epidermal response to wound injury, where a defective response leads to poor wound healing.

VITAMIN D AND CALCIUM SIGNALING IN INJURY INDUCED ACTIVATION OF EPIDERMAL SC THROUGH INTERACTION WITH β-CATENIN SIGNALING

Studies of the role of VDR in skin have previously focused on hair cycling as alopecia (hair loss) is a striking phenotype in global *Vdr* knockout (KO) mice[28], in which a gradual decrease in bSC has been reported accompanied with impaired β-catenin signaling[18]. Delayed wound healing is reported in these mice, but it was primarily attributed to altered dermal fibroblast function not to alterations in epidermal function[29]. However, our studies showed that VDR has an essential role in epidermal SCs and progeny during cutaneous wound healing. Wound closure is delayed and wound re-epithelialization is retarded in *Vdr* conditional knockout (cKO) mice in which *Vdr* is specifically removed from *Krt14* expressing epidermal SCs and progeny[30,31]. The number of SCs residing in the IFE as well as the HF is decreased[30] demonstrating impaired self-renewal of these SCs. In addition, *Vdr* cKO results in blunted SC proliferation and in impaired β-catenin signaling[30] that plays an important role in the epidermal response to wounding. These results are observed only when *Vdr* cKO mice are maintained on a low dietary calcium. Therefore, we explored the co-operative role of calcium signaling with vitamin D signaling by generating conditional double knockout mice (cDKO), in which both *Vdr* and *Casr* are deleted from *Krt14* expressing epidermal SCs and progeny[32]. Delayed wound closure and retarded wound re-epithelialization is also observed in these cDKO mice ingesting normal calcium diets. Injury activated SC proliferation is impaired at the wounding edges. In addition, injury induced induction of β-catenin target genes was also blunted in cDKO wounds[32]. These responses are similar to skin wounds in *Krt14* specific *β-catenin* KO mice. These results show that *Vdr* and *Casr* are essential for injury-induced SC activation at least in part *via* stimulation of β-catenin signaling.

VITAMIN D AND CALCIUM SIGNALING IN ADHERENS JUNCTION FORMATION ESSENTIAL FOR EPIDERMAL REMODELING

Our transcriptomic study also revealed that AJ signaling is a top canonical pathway altered in the epidermis of mice that lack both *Vdr* and *Casr* (cDKO) in *Krt14* expressing keratinocytes[32]. The expression levels of E-cadherin and the levels of the epithelial specific desmosome component desmoglein 1 are decreased in wounds of cDKO mice[32]. In addition, the expression of the IFE early differentiation marker KRT1, middle differentiation marker IVL, and late differentiation marker LOR in the epidermis did not extend across the wound and remained disorganized in the shortened epithelial tongues at the remodeling stage of wound healing of *Vdr* cKO[30]. The reduction of E-cadherin and differentiation markers are also observed in *Casr* cKO mice, in which *Casr* is removed from epidermal SC and progeny[33], in association with a delay in wound closure and reduced wound re-epithelialization. Similarly, cell migration is impaired in an *in vitro* wound scratch model when *VDR*[30] , *CaSR*[33] or both *VDR* and *CaSR*[32] are silenced in cultured human keratinocytes. Therefore, vitamin D and calcium signaling are critical for keratinocyte migration and E-cadherin/catenin mediated epidermal differentiation, each essential for epidermal regeneration during wound healing.

POTENTIAL MECHANISMS FOR VITAMIN D AND CALCIUM SIGNALING TO REGULATE WOUND RE-EPITHELIALIZATION

Compensatory and/or interacting aspects of vitamin D and calcium signaling on wound healing exist. We examined two processes; (1) β-catenin signaling, that induces epidermal proliferation to produce the cells that subsequently regenerate the epidermis[4,5], and (2) the E-cadherin/catenin complex that is critical for keratinocyte differentiation as well as migration during wound induced re-epithelialization[34]. Our working model summarizes our findings illustrating the reduction in proliferation due to defects in the nuclear actions of β-catenin and decreased re-epithelialization caused by failure of migration, differentiation and formation of the AJ in the epidermis of mice in which vitamin D and calcium signaling are disrupted (Figure 1). Potential molecular mechanisms by which vitamin D and calcium interact to control migration and proliferation of keratinocytes during wound induced epidermal regeneration are shown (Figure 2). First, VDR induces genes including CaSR and β-catenin regulating differentiation genes in the epidermis and HFs[11]. VDR may facilitate β-catenin binding to its response elements in target genes such as cyclin D1 to promote cell proliferation[16]. VDR also is required for calcium, mediated by the CaSR, to form E-cadherin/catenin complex to stimulate AJ signaling[22]. This is due in part to activation of the Src/Fyn kinases that phosphorylate the catenins facilitating their incorporation into the E-cadherin/catenin complex. This complex provides a reservoir of β-catenin in the membrane. The shift of β-catenin from the nucleus to the membrane is crucial to allow differentiation, in part by reducing its proliferative function in the nucleus.

The E-cadherin/catenin complex includes α-catenin, that links the complex to the cytoskeleton subsequently enabling cells to migrate. The complex also includes the enzymes phosphatidyl inositol 3 kinase (PI3K) and phosphatidyl inositol 4-phosphate 5-kinase 1α (PIP5K1α)[34,35]. These enzymes sequentially phosphorylate of PIP and PIP2 to PIP3 to activate PLC-γ1 and other signaling molecules such as Akt. PLC-γ1 cleaves PIP2 to form IP3 and DAG; IP3 releases intracellular [Ca]i from intracellular stores, which is essential for the acute response to wounding, and DAG which along with calcium activates PKCα. PKC regulates the activity of AP-1 transcription factors involved in differentiation. Moreover, PKCα phosphorylates desmoplakin, a component of desmosomes, that loosens their intercellular adhesion to enable keratinocyte migration across the wounds[36].

Efficient wound repair is critical for life by restoring the integrity of the skin to prevent the invasion by infectious organisms and other harmful materials and the loss of body fluids. Understanding the mechanisms by which the SC populations in the skin respond to wounding should lead to better therapies to promote more efficient healing. Examining the role of vitamin D and calcium signaling in this process is an important step in this direction. Moreover, our studies address the more general question of tissue regeneration, both pathologic as in chronic wounds and physiologic as in normal wound repair in the skin and other tissues. The roles of β-catenin and E-cadherin signaling in SC as they are regulated by vitamin D and calcium during the

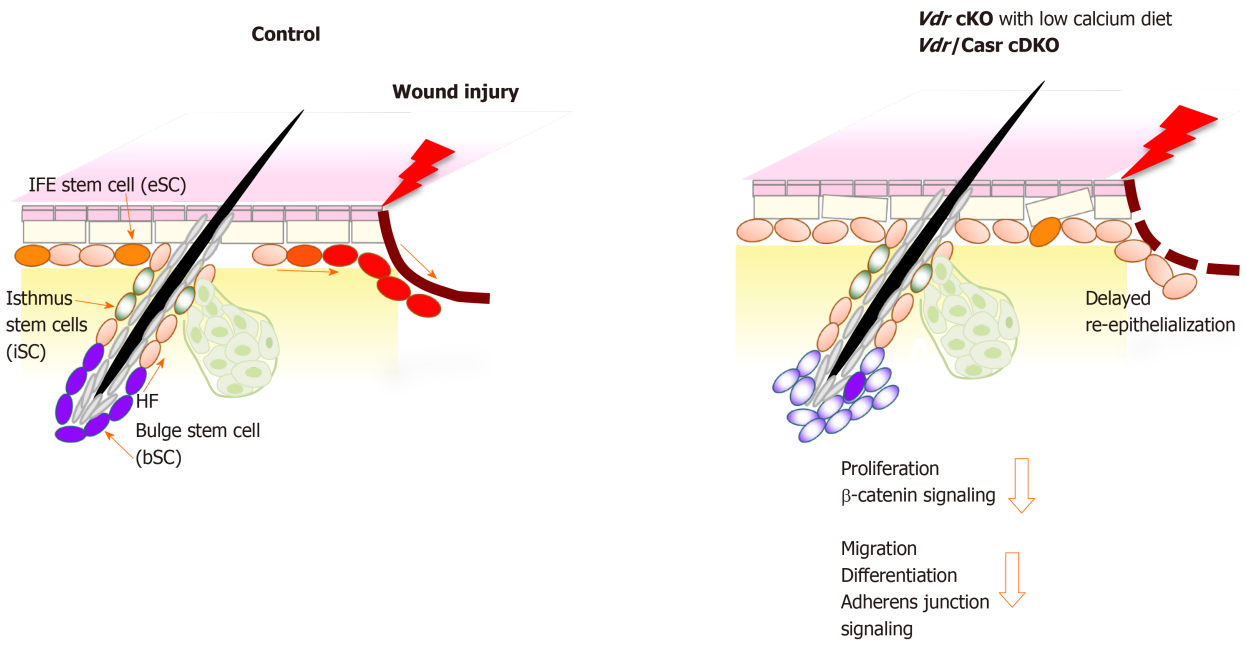

Figure 1 Schematic model showing that deficiency in both vitamin D receptor and calcium sensing receptor prevents proliferation and migration of keratinocytes thus delaying wound re-epithelialization of the wounded epidermis. The location of the different stem cells (SC) niches in skin is shown; stem cells in the hair follicle bulge (bSC shown in purple color) required for hair cycling, isthmus stem cells (iSC, green) in the junctional zone of the upper hair follicle responsible for sebaceous gland renewal, and epidermal stem cells (eSC, orange) in the interfollicular epidermis (IFE) responsible for epidermal regeneration. Upon injury of normal skin (left panel), these stem cells at the wound edge are activated to proliferate, shifting their normal cell fate to re-populate the disrupted epidermis by migrating to the wound and differentiating to re-epithelialize the wound (red color). In contrast, vitamin D receptor (*Vdr*) conditional knockout (cKO) mice fed a calcium deficient diet and *Vdr/Casr* double KO (cDKO) mice show defects in these stem cells that reduce their responses to wounding (right panel). The number of bSC and eSC decreases in *Vdr* cKO demonstrating defects in their self-renewal. Both *Vdr* cKO and cDKO mice have decreased injury induced proliferation of these stem cells associated with a reduction in β-catenin signaling. Delayed re-epithelialization is accompanied with defects in migration and differentiation of these stem cells, mediated by decreased AJ signaling. cKO: Conditional knockout; cDKO: Conditional double knockout.

response to wounding and epidermal remodeling are central to the wounding response. Further study of the shift in the transcriptional profile during wounding as affected by deletion of *Vdr* and/or *Casr* is likely to reveal a better understanding of the molecular mechanisms by which VDR and CaSR sequentially regulate the different aspects of the wounding response with the potential that these results will lead to new approaches to treatment of chronic wounds.

These studies have clinical significance as vitamin D deficiency is linked with poor wound healing[2,3]. Improved wound healing of patients with diabetic foot ulcers with vitamin D supplementation compared to placebo is supported by a randomized clinical trial of oral vitamin D supplementation[37]. Likewise, calcium alginate dressings are superior to other wound care products[38] indicating the clinical role of calcium signaling in wound repair. Like vitamin D signaling, calcium signaling is expected to play an important role for activation, migration and differentiation of the SCs regenerating the epidermis.

CONCLUSION

In summary, we have discussed the role of vitamin D and calcium signaling in epidermal SCs and progeny essential for normal wound re-epithelialization in the epidermis. We propose that vitamin D and calcium promote wound re-epithelialization, through both β-catenin and AJ signaling.

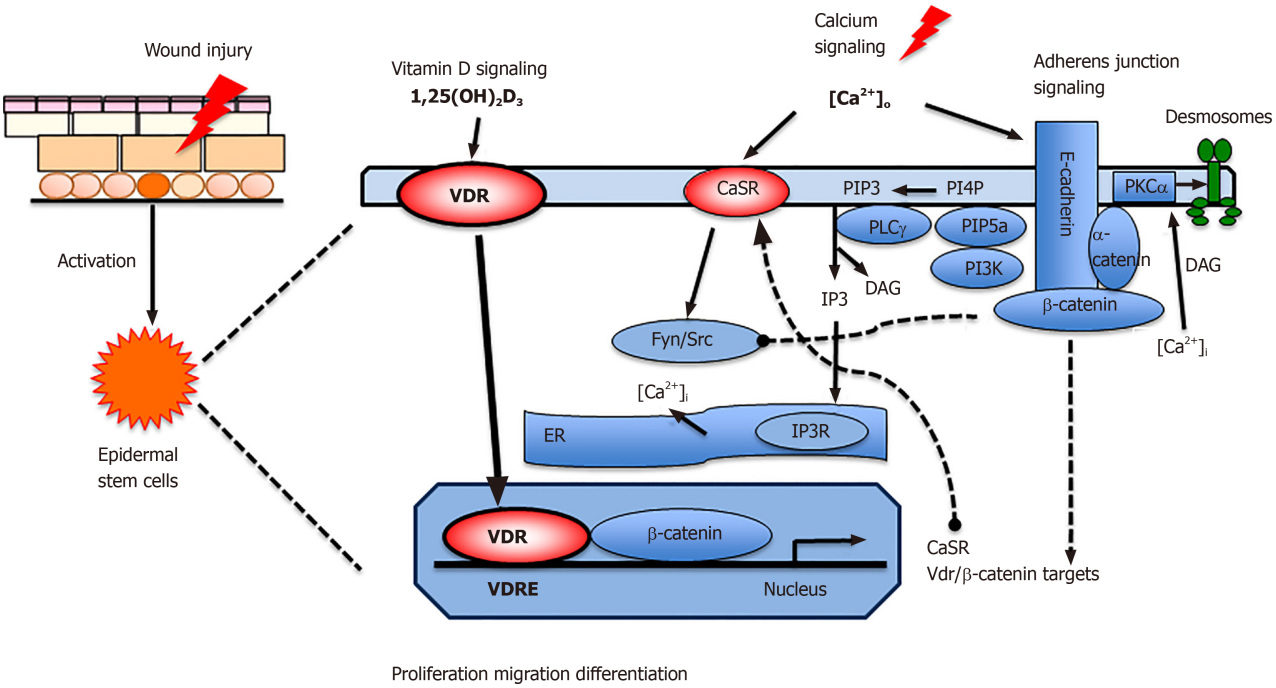

Figure 2 Proposed model in which vitamin D and calcium signaling mutually regulate β-catenin and AJ signaling essential for wound re-epithelialization. First, vitamin D receptor (VDR) may partner with β-catenin in the nucleus to regulate the expression of β-catenin target genes such as Cyclin D1 to promote proliferation of stem cells when the skin is wounded. Subsequently extracellular calcium [Ca]o in collaboration with VDR stimulates E-cadherin/catenin complex formation to promote keratinocyte differentiation while reducing the proliferative stimulus by sequestering β-catenin in the membrane. The E-cadherin/catenin complex formation is facilitated by the activation of Fyn/Src kinases by the CaSR which phosphorylate the catenins required for their recruitment into the E-cadherin/catenin complex. The E-cadherin/catenin complex not only provides a reservoir of β-catenin in the membrane but also includes a link to the cytoskeleton *via* a catenin enabling cell migration and differentiation essential for epidermal remodeling during wound re-epithelialization. Moreover, enzymes within the E-cadherin/catenin complex sequentially phosphorylate PIP to PIP3, that activates PLCγ, that in turn hydrolyzes PIP2 to DAG and IP3. The latter stimulates the IP3 receptor in subcellular organelles (ER and Golgi in keratinocytes) to release calcium. DAG, on the other hand along with calcium activates PKCa, the enzyme that activates the AP-1 transcription factors involved in the expression of differentiation markers in keratinocytes as well as phosphorylation of desmoplakin, which alters the desmosomal structure facilitating migration of the keratinocytes to re-epithelialize the wounds. VDR: Vitamin D receptor; CaSR: Calcium sensing receptor; DAG: Diacylglycerol;ER: Endoplasmic reticulum; PLCγ: Phospholipase γ; IP3: Inositol trisphosphate; PIP3: Phosphatidylinositol 3,4,5-trisphosphate; PI3K: Phosphatidyl inositol 3 kinase; PIP5K1a: phosphatidyl inositol 4-phosphate 5-kinase 1α.

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MINIREVIEWS

Adipose-derived stem cell therapy shows promising results for secondary lymphedema

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Abstract

Lymphedema is mainly identified by progressive soft tissue swelling in impaired lymphatic system. Secondary lymphedema attributed to cancer therapy, parasite infection, and trauma remains a serious global disease. Patients with lymphedema suffer swelling, pain, and fatigue, with the dysfunction of the deformed extremities reducing the quality of life and increasing the risk of infection and lymphangiosarcoma. Adipose-derived stem cells (ADSCs) possess prominent regenerative potential to differentiate into multilineage cells, and produce various lymphangiogenic factors, making ADSC therapy a promising approach for lymphedema. The development of lymphedema consists of local inflammation, the fibrosis of lymphatic vessels, and the deposition of adipose fat. Existing animal models do not mimic the chronic inflammation environment, therefore suitable models are required in further studies. Some signal pathways and molecular mechanisms in physiological and pathological lymphagiogenesis remain unclear. In previous animal and human trials, ADSC therapy reduced edema in varying degrees. A larger number of trials with larger samples and longer follow-up periods are required to verify the efficiency and feasibility of ADSC therapy. ADSCs are of easy availability and immune exemption, making them a candidate for lymphedema treatment. Whether ADSCs enhance malignant characteristics or trigger the malignant change deserves further exploration and study before ADSC therapy can be made widely available.

Key Words: Secondary lymphedema; Adipose-derived stem cells; Lymphangiogenesis; Stem cells; Cell therapy; Lymphatic regeneration

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Core tip: Secondary lymphedema attributed to cancer therapy, parasite infection, and trauma remains a serious global disease. Adipose-derived stem cells (ADSCs) possess prominent regenerative potential to differentiate into multilineage cells, and produce various lymphangiogenic factors, making ADSC therapy a promising approach for lymphedema. However, suitable animal models are required for further studies and a larger number of clinical trials are necessary to verify the efficiency and feasibility of ADSC therapy. This review exhibits the pathophysiology of lymphedema and elaborates how ADSCs can improve lymphatic function.

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INTRODUCTION

Secondary lymphedema remains a serious global disease with primary lymphedema due to genetic defects accounting for only a subset of those afflicted[1,2]. The common causes of secondary lymphedema are attributed to cancer therapy, parasite infection, and trauma[3]. Most sufferers are those with cancer who require radiotherapy or lymph node dissection[4]. For example, the development of breast cancer–related lymphedema results from axillary lymph node dissection, chemotherapy, radiotherapy, and postoperative complications[5]. Patients with lymphedema suffer swelling, pain, and fatigue, with the dysfunction of the deformed extremities reducing the quality of life and increasing the risk of infection and lymphangiosarcoma[4].

Conservative treatments include pharmacotherapy and physiotherapy (compression and lymphatic drainage massage), whilst surgical treatments include lymphaticovenular anastomosis, lymph node transfer, fluid drainage, and liposuction[6-8]. However, no effective treatments for lymphedema are available and impairment during surgical therapy can reversibly aggravate lymphedema.

In the last decade, cell-based therapies have emerged as a research hotspot due to their capacity to promote tissue regeneration. Mesenchymal stem cells (MSCs) are multipotent adult progenitor cells with favorably low immunogenicity and unique regenerative potential[9-11], making them a therapeutic option for lymphatic regeneration. MSCs include three major stem cell types, namely, bone marrow-derived MSCs (BM-MSCs), umbilical cord-MSCs (UC-MSCs), and adipose-derived stem cells (ADSCs). ADSCs have attracted increased attention due to their ease of accessibility, avoidable ethical concerns, and adequate sources. ADSCs grow stably[12] and produce various lymphangiogenic factors such as vascular endothelial growth factor C (VEGF-C), making ADSC therapy a promising approach for diseases of the lymphatic system[11].

In this review, we discuss the basic information regarding the pathophysiology of lymphedema including local inflammation, fibrosis, and the deposition of adipose tissue (AT). We discuss the development of lymphedema and outline how ADSCs can improve lymphatic function. Previous studies associated with ADSC-based therapy for the treatment of lymphedema are discussed, including both animal and human studies, with a specific focus on the outcomes of ADSC therapy in the clinic. The focus of this review is to explore the efficiency and feasibility of ADSC-based therapy. In addition, the future perspectives of ADSCs in the field of lymphatic regeneration are discussed.

EMBRYONIC LYMPHANGIOGENESIS

Lymphatic endothelial cells specification

Lymphatic specification can be observed from embryonic day 9.5 (E9.5) in a subset of cells in the walls of the cardinal veins. VEGF-C and its receptor vascular endothelial growth factor receptor 3 (VEGFR3) comprise the most essential signaling pathways during initial lymphatic development, in addition to lymphatic endothelial cell (LEC) proliferation, migration, and maintenance during early embryonic growth[13-15].

Prospero homeobox protein 1 (Prox1) determines the fate of differentiation[16,17], and is a master lymphatic transcription factor during cell proliferation and the maintenance of lymphatic integrity. In *Prox1* knockout mice, LEC budding is observed during the early stages of development, suggesting that the early expression of lymphatic vascular hyaluronan receptor - 1 (Lyve-1) together with that of Prox1 represents the first indication of lymphangiogenesis[18]. Coup-TFII directs the polarized expression of Prox1 in endothelial cells within the cardinal vein[19]. Notch signaling regulates normal lymphatic vessel patterning, the deletion of which leads to excessive Prox1+ LEC differentiation and lymphatic overgrowth[20]. LEC precursors migrate out of the vein under the control of the VEGF-C/VEGFR-3/Prox1 axis[21].

Lymphatic sprouting growth

Mature lymphatic structures are composed of capillaries, pre-collectors, and collecting vessels, and rely on the formation of lymph sacs and lymphatic plexuses. At approximately E10.5, Prox1 + LECs begin to migrate. At E11.5, VEGF-C/VEGFR-3 signaling stimulates lymph sac morphogenesis, and its hyperactivation leads to the overgrowth of lymph sacs[22].

Lymphatic maturation

Lymph sacs and lymphatic plexuses undergo further remodeling to form a functional lymphatic vessel network from E15.5 to early post-birth stages[23]. The formation of lymphatic valves, the recruitment of mural granulosa cells, and the deposition of the basement membrane are signs of maturity for collecting vessels. The transition of the LEC junctions from zippers to buttons characterizes the process of lymphatic capillary maturation[24].

PATHOLOGICAL CHANGES DURING LYMPHEDEMA

The pathophysiology of lymphedema remains poorly understood due to the lack of suitable animal models[25,26]. Rodent tails and hindlimb models fail to accurately recapitulate latent onset in the human body, which ranges from 3 mo to 3 years[27,28]. However, a positive feedback loop is widely accepted during lymphedema development, involving local inflammation, the fibrosis of lymphatic vessels, and the deposition of adipose fat[29,30]. Lymphedema is therefore chronic, potentially progressive, and irreversible.

Local chronic inflammation and pathological lymphangiogenesis

In animal models of lymphedema, the infiltration of lymphocytes and macrophages is observed. Lymphatic function can be improved through immunosuppressive drug targeting at T cells, including tacrolimus[31] and atorvastatin[27]. In contrast to acute inflammation, active T cells play an important role in the progressive development of lymphedema including neolymphatic vessel formation and fibrosis[4,32]. CD4+ cells play a key role in impaired lymphangiogenesis and lymphatic dysfunction, whilst the depletion of CD8+ cells has minimal effects[33]. *CD4* knockout mice with acquired lymphedema exhibit lower levels of swelling and improved lymphatic function. The number of CD4+ cells is also positively associated with the severity of edema[4]. However, CD4+ T cells have different roles when cooperating with macrophages. Ogata *et al*[27] found that the addition of CD4+ T cells had no effect on tube formation. However, when CD4+ T cells were co-cultured with macrophages, new lymphatic tubes were observed. Macrophages are essential during lymphangiogenesis. Recent studies show that RAMP1 signaling accelerates lymphedema by inhibiting the recruitment of macrophages[34].

T cell-derived cytokines including interleukin (IL)-4, IL-13, IL-17, interferon gamma (IFN-γ), and transforming growth factor (TGF)-β1 negatively regulate lymphangiogenesis. *In vitro*, IL-4, IL-13, and IL-17 have been shown to inhibit LEC proliferation through the downregulation of LEC genes[35]. IL-4, IL-13, IL-17, and TGF-β1 participate in the development of fibrosis-related diseases[4]. IFN-γ and IL-17 activate macrophages through VEGF-C production during lymphangiogenesis in different disease models. IFN-γ and IL-17 can activate macrophages and enhance VEGF-C production during lymphangiogenesis of different disease models[27].

A chronic inflammatory environment initiates diverse lymphangiogenesis processes[32]. Lymphatic vessels do not reform spontaneously, and the remodeling of pre-existing vessels occurs, leading to the dilation of vessels and decreased contractile frequencies[36,37]. It remains unclear how VEGF-C/VEGFR-3 signaling regulates

lymphedema-induced lymphangiogenesis.

Pathological changes of adipose tissue

A stable lymphatic system is critical for homeostasis, immunity, and lipid reabsorption[33]. Adipocytes are the main parenchymal cells in AT that contribute to energy storage and pathogen defense. Adipocytes are sensitive to pathological changes such as inflammation[36]. The accumulation of lymphatic fluid that contains free fatty acids can lead to fat deposition by activated adipocytes, upregulating fat differentiation genes[24,35,38]. Enhanced lipid storage leads to the hypertrophy and hyperplasia of adipocytes. In AT samples from lymphedema patients, a decrease in elastic fibers and an increase in collagen fibers are observed[30]. Active adipogenesis and fibrosis alter the physiological structure of AT.

ADSCs are multipotent cells with the potential to differentiate into multilineage cells including osteocytes, myocytes, chondrocytes, adipocytes, astrocytes, and endotheliocytes *in vitro* and *in vivo*[39]. However, significantly fewer stem cells exist in pathologic AT compared to normal AT. The differentiation potential of ADSCs to adipocytes can compensate for inadequate lipid storage capacity[40]. Lymphedema leads to the consumption of anti-inflammatory macrophages, which play an important role in the prevention of inflammation and tissue repair. Conversely, inflammatory macrophages are prevalent in hypertrophic AT[34].

ADSC-BASED THERAPIES

We searched PubMed, ClinicalTrials.gov, and EMBASE for published articles on lymphedema. "ADSCs", "stem cell", "cell therapy", and "lymphedema" were used as the main search terms. All relevant studies performed from November 2009 to 2019 were selected. In total, five articles reported animal experiments (Table 1) and four reported human experiments (Table 2). In all studies, the location of lymphedema, cell origin, injection methods, evaluation methods, and the results were analyzed.

Animal studies

Mice are the only used animal models for lymphedema, which occurs in either the hindlimbs or tails as a result of circumferential incision. Irradiation is used as an auxiliary method with circumferential incision[41,42]. ADSCs are harvested from the AT of the same species from both intraabdominal and inguinal regions. Hwang *et al*[43] used ADSCs isolated from the human body, and complications related to rejection reactions were not observed. VEGF-C hydrogel sheets when applied to the injection site can reduce edema 3 to 4 d post-treatment. The reduction in the circumference and volume at the edema site following the injection of ADSCs occurs within 2-4 wk of treatment.

The dose of cells injected varied from 1×10^4 to 2×10^6, and all showed improved lymphatic function. Yoshida *et al*[42] divided mice into groups injected with 1×10^4, 1×10^5, and 1×10^6 ADSCs. The number of lymphatic vessels significantly increased at 2 wk in a dose dependent manner. Increased LYVE-1 expression with a treatment dose of 1×10^6 cells was significantly higher than that with treatment doses of 1×10^5 and 1×10^4 cells. Likewise, a higher dose of 1×10^5 showed significantly greater LYVE-1 expression than the dose of 1×10^4. Stem cells were subcutaneously injected at the site of lymphedema. Shimizu *et al*[44] showed that ADSCs stimulate lymphangiogenesis by secreting VEGF-C and through the recruitment of lymphatic endothelial progenitor cells. Ackermann *et al*[45] reported that ADSC therapy promotes lymphangiogenesis and lymphedema but to lower levels than platelet-rich plasma (PRP).

Human studies

Peña Quián *et al*[46] provided a case report on a patient with edematous lower limbs resulting from recurrent lymphangitis. Autologous ADSCs ($1\text{-}2.2 \times 10^9$) were injected and a greater number of new lymphatic ramifications and lymph nodes were observed at 6 mo post-treatment *via* lymphoscintigraphy. Toyserkani *et al*[47] used autologous ADSCs in a patient suffering breast cancer–related lymphedema with deformed upper limbs. Fat grafting was performed at the same time. A total of eight axilla injections were performed at a total dosage of 4.0×10^7 cells. The time of follow-up was 4 mo, and positive outcomes were observed. The reduction of arm volume along with the decrease in heaviness and tension led to a lower requirement for compression therapy. In 2017, Toyserkani *et al*[48] enrolled ten patients to explore the feasibility and safety of ADSC therapy. Patients received the same treatment at a slightly higher dose of 5×10^7

Table 1 Adipose-derived stem cell-based therapy for secondary lymphedema in animals

Year	Ref.	Animal number	Edema site	Lymphoedema modeling	ADSC origin	Cell dose	Injection method	Auxiliary treatment	Valuation criteria	Result(s)
2011	Hwang *et al*[43]	*n* = 5 (5 groups)	Hindlimb	Circumferential incision	Human	NM	Injected subcutaneously at the site of the damaged lymphatic vessels	VEGF-C hydrogel (A VEGF-C hydrogel sheet was applied to the injection site and was sutured into the injured dermal junction)	(1) Circumference; and (2) IHC staining (LYVE-1, PKH-26)	(1) A significant decrease in dermal edema at 3-4 wk; and (2) Significant lymphatic vessel regeneration
2012	Shimizu *et al*[44]	*n* = 12 (3 groups)	Tail	Circumferential incision	Allogeneic	2×10^6	Injected at two different points of the lymphedematous skin flap	N	Circumference	A decrease in lymphedema
2015	Ackermann *et al*[45]	*n* = 10 (3 groups)	Tail	Circumferential incision	Allogeneic	NM	NM	N	(1) Circumference; (2) IHC staining (LYV-1); and (3) Real-time laser Doppler imaging for wound perfusion	(1) ADSCs affected lymphangiogenesis and lymphedema development; and (2) PRP affected more significantly than ADSCs
2015	Yoshida *et al*[42]	*n* = 20 (5 groups)	Hindlimb	Circumferential incision	Allogeneic	1×10^4; 1×1^5; 1×10^6	NM	N	(1) Circumference; and (2) IHC (LYVE, VEGF-C,VEGFR, EGFP)	The number of lymphatic vessels significantly increased at 2 wk, which was dose-dependent of implanted ADSCs
2017	Hayashida *et al*[41]	*n* = 5 (4 groups)	Hindlimb	Circumferential incision	Allogeneic	1×10^6	Injected subcutaneously at proximal and distal limb	N	(1) Hind-paw edema volume; and (2) IHC (LYVE-1, VEGF-C)	Increased the number of lymphatic vessels; induced the lymphatic flow drainage to the circulatory system

ADSCs: Adipose-derived stem cells; IHC: Immunohistochemistry; LYVE-1: Lymphatic vascular hyaluronan receptor-1; Prox-1: Prospero homeobox protein 1; PRP: Platelet-rich plasma; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; NM: Not mentioned; N: None.

cells. However, volume reduction was not significant after 6 mo of follow-up. Up to 50% of the patients reported an alleviation of their discomfort and had a lower requirement for conservative management. In 2019, Toyserkani *et al*[49] performed lymphoscintigraphic evaluations after one year of follow-up. No changes in arm volume and only mild transient complications related to liposuction were noted.

CONCLUSION

Stem cells can differentiate into multilineage cells that exist in nearly all tissues and organs. In theory, damaged tissues and organs can recover after stem cell implantation. Secondary lymphedema affects millions with sufferers experiencing persistent, uncomfortable, and dysfunctional extremities. However, existing therapies including conservative and surgical methods fail to improve lymphatic function.

ADSCs can be isolated from ATs by mildly invasive procedures. A prominent characteristic of ADSCs is their low immunogenicity, due to the low levels of

Table 2 Adipose-derived stem cells-based therapy for secondary lymphedema in human

Year	Ref.	Study type	Patient number	Edema site	Etiology	ADSC origin	Cell dose	Injection method	Auxiliary treatment	Valuation criteria	Result(s)	Follow-up
2015	Peña Quián *et al*[46]	Case report	1	Lower limb	Recurrent lymphangitis	Autologous	1-2.2 × 10^9	NM	N	Lymphoscintigraphy	The presence of new lymphatic ramifications and a greater number of lymph nodes	6 mo
2016	Toyserkani *et al*[47]	Nonramdomized clinical trial	1	Upper limb	BCRL	Autologous	4.07 × 10^7	Injected at 8 different points of axilla	Fat grafting (10 mL)	(1) Circumference; (2) DXA scans; and (3) Discomfort (infection, pain, and swelling)	(1) A reduction in volume; (2) Relief of symptoms (heaviness and tension); (3) A reduction of compression therapy; and (4) No complication	1 and 4 mo
2017	Toyserkanl *et al*[48]	Nonramdomized clinical trial	10	Upper limb	BCRL	Autologous	5 × 10^7	Injected at 8 different points of axilla	Fat grafting (28 mL)	(1) Volume assessment; (2) DXA scans; and (3) Discomfort (redness, swelling, itching, pain, and infection)	No significant reduction in volume; reduction of conservative managements (50%); reduction of symptoms	1, 3, 6, and 12 mo
2019	Toyserkanl *et al*[49]	Nonramdomized clinical trial	10	Upper limb	BCRL	Autologous	5 × 10^7	Injected at 8 different points of axilla	Fat grafting (30 mL)	(1)Volume assessment; (2) DXA scans; and (3) Discomfort (redness, swelling, itching, pain, and infection)	No significant reduction in volume; reduction of conservative managements (50%)	1, 3, 6, and 12 mo

ADSCs: Adipose-derived stem cells; BCRL: Breast cancer–related lymphedema; NM: Not mentioned; N: None.

expression of major histocompatibility complex (MHC) and costimulatory molecules[50]. ADSCs produce immunomodulatory cytokines including TGF-β that block IFN-γ-induced MHC expression[51]. The downregulation of MHC can avoid immune surveillance, producing immune-privileged cells ADSCs[52]. Complications relating to immune rejection are therefore sparse. ADSCs remain stable over long passages and can differentiate with low rates of apoptosis. As such, ADSC-based therapy may play an important role in secondary lymphedema. ADSCs can differentiate into progenitor cells for lymphangiogenesis and secrete VEGF-C. Both animal and human studies show positive outcomes after the injection of ADSCs with minimal complications. ADSC-based therapy is therefore promising for the treatment of secondary lymphedema.

However, some issues remain for ADSC-based therapy. Suitable animal models are required as pathological changes in acute inflammation differ from those of chronic inflammation, producing variable therapeutic outcomes. Second, a larger number of clinical trials with larger samples and longer follow-up periods are required. In addition, the safety of ADSC-based therapy should be assessed. In lung cancer models[53], ADSCs interact with LLC1 cells through their ability to secrete IL-6 and enhance malignant characteristics *in vitro* and *in vivo*. We believe that ADSC-based therapy is therefore key to the future treatment of secondary lymphedema.

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ORIGINAL ARTICLE

Basic Study

Involvement of glycated albumin in adipose-derived-stem cell-mediated interleukin 17 secreting T helper cell activation

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Author contributions: Pestel J performed the experiments, acquired and analyzed data and was involved in data interpretation and in the writing of the manuscript; Robert M provided the residual adipose tissues and participated in the writing of the manuscript; Corbin S supervised the statistical data and helped in the revision of the manuscript; Vidal H participated in the design of the study, data interpretation, and writing of the manuscript; Eljaafari A designed, coordinated the study, was involved in data interpretation and in the writing of the manuscript. All authors approved the final version of the article.

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Abstract

BACKGROUND

Advanced glycation end products (AGE) are a marker of various diseases including diabetes, in which they participate to vascular damages such as retinopathy, nephropathy and coronaropathy. Besides those vascular complications, AGE are involved in altered metabolism in many tissues, including adipose tissue (AT) where they contribute to reduced glucose uptake and attenuation of insulin sensitivity. AGE are known to contribute to type 1 diabetes (T1D) through promotion of interleukin (IL)-17 secreting T helper (Th17) cells.

AIM

To investigate whether lean adipose-derived stem cells (ASC) could be able to induce IL-17A secretion, with the help of AGE.

METHODS

As we have recently demonstrated that ASC are involved in Th17 cell promotion when they are harvested from obese AT, we used the same co-culture model to

standards. The Committee for people Protection emanating from the Research Ministry, has given us its approval for the use of human adipose tissues isolated from residues of visceral surgery. Informed consents were signed by each donor.

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measure the impact of glycated human serum albumin (G-HSA) on human lean ASC interacting with blood mononuclear cells. IL-17A and pro-inflammatory cytokine secretion were measured by ELISA. Receptor of AGE (RAGE) together with intercellular adhesion molecule 1 (ICAM-1), human leukocyte Antigen (HLA)-DR, cluster of differentiation (CD) 41, and CD62P surface expressions were measured by cytofluorometry. Anti-RAGE specific monoclonal antibody was added to co-cultures in order to evaluate the role of RAGE in IL-17A production.

RESULTS
Results showed that whereas 1% G-HSA only weakly potentiated the production of IL-17A by T cells interacting with ASC harvested from obese subjects, it markedly increased IL-17A, but also interferon gamma and tumor necrosis factor alpha production in the presence of ASC harvested from lean individuals. This was associated with increased expression of RAGE and HLA-DR molecule by co-cultured cells. Moreover, RAGE blockade experiments demonstrated RAGE specific involvement in lean ASC-mediated Th-17 cell activation. Finally, platelet aggregation and ICAM-1, which are known to be induced by AGE, were not involved in these processes.

CONCLUSION
Thus, our results demonstrated that G-HSA potentiated lean ASC-mediated IL-17A production in AT, suggesting a new mechanism by which AGE could contribute to T1D pathophysiology.

Key Words: Interleukin 17 secreting T helper cells; Lean adipose tissue; Type 1 diabetes; Advanced glycation end products; Adipose-derived stem cells

Core tip: Using a coculture model with human lean adipose-derived stem cells (ASC) and mononuclear cells, we have shown in this study that glycated human serum albumin (G-HSA) enhances lean ASC-mediated interleukin (IL)-17A, interferon gamma and tumor necrosis factor alpha secretion. This effect involved the advanced glycated end products (AGE)/Receptor of advanced glycated end products (RAGE) axis as assessed by anti-RAGE blocking antibodies and was associated with increased expression of RAGE and human leukocyte antigen-DR molecules. Thus, our results demonstrated that G-HSA potentiated lean ASC-mediated IL-17A production in adipose tissues, suggesting a new mechanism by which AGE could contribute to type 1 diabetes pathophysiology.

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INTRODUCTION

Glycated proteins result from non-enzymatic Maillard reactions between sugars and amine residues, mostly lysine and arginine[1]. While in the healthy body all proteins can be modified by non-enzymatic glycation reactions, advanced glycation end products (AGE) are known to exert deleterious effects on human health when they are too abundant, as observed in diabetes, arteriosclerosis, renal failure and also in Alzheimer, and Parkinson diseases[2-4]. Although glycated haemoglobin is a major biomarker for diabetes mellitus diagnosis[5], the role of glycated albumin as a potential diagnostic marker[6] is currently under investigation, due to the higher levels of albumin in blood, its shorter life, and its independence from haemolytic processes[7-9]. In addition to modifications of protein structure and function, AGE pathogenic effects mostly result from binding and activation of specific receptors, named receptor of advanced glycated end products (RAGE)[10,11]. Those receptors belong to the immunoglobulin superfamily of transmembrane proteins[12]. Besides AGE, RAGE can

bind a variety of molecules, such as the high mobility group box-1, the β-amyloid peptide and the S100/calgranulin[13]. Interaction of RAGE ligands with RAGE, initiate a cascade of signalization leading to activation of $p21^{ras}$, p44/p42 mitogen-activated protein kinases and nuclear factor-kappa B (NFKB), which generally results in the synthesis of proinflammatory cytokines[14-16]. The implication of AGE/RAGE in diabetes pathophysiology has been demonstrated using RAGE blockade experiments able to inhibit diabetes dysfunctions in vessels or in organs, while AGE injection in mice provoked such dysfunctions[17-19].

T-lymphocytes play an important role in diabetes, either through activation of auto-immune cells directed against beta-pancreatic cells in the case of type 1 diabetes (T1D), or through infiltration of tissues or organs such as adipose tissue (AT) in type 2 diabetes (T2D). In T1D, contribution of AGE/RAGE to diabetes evolution has been clearly demonstrated. For example, RAGE blockade experiments prevented diabetes transfer with diabetogenic T cells in non-obese diabetic/severe combined immuno-deficiency mice[20]. Moreover, T cells from T1D patients or from at risk diabetes relatives, have been shown to express elevated levels of intra-cellular RAGE associated with increased T cell survival and inflammatory cytokine release[21]. AGE/RAGE interaction is also known to play a role in interleukin (IL)-17 immune responses as shown by AGE-mediated up-regulation of RAGE expression in T cells of T1D patients, which resulted in increased IL-17A secretion[22].

The interleukin 17 secreting T helper (Th17) cell subset has been recently discovered as a T-cell inflammatory lineage that mainly secretes IL-17A and IL-17F cytokine whose receptors are ubiquitously expressed[23]. Those receptors are able to spread inflammation due to their ability to activate secretion of pro-inflammatory cytokines and metalloproteinases following IL-17A binding[24].

We have recently implicated adipose-derived-stem cells (ASC) and adipocytes (AD) in the promotion of Th17 cells through cell-to-cell contact-dependent interactions with blood mononuclear cells (MNC)[25,26]. This function was likely to be mostly displayed by ASC obtained from obese rather than from lean individuals and resulted in inhibiting adipogenesis and insulin response of obese ASC and AD, respectively. In the present study, we aimed to determine the potential role of the AGE/RAGE axis on ASC-mediated Th17 promotion in lean individuals. Therefore, we investigated herein whether glycated albumin would induce IL-17A secretion by T cells, and whether anti-RAGE monoclonal antibody (mAb) would prevent this activation. To this purpose, we co-cultured lean ASC with MNC and treated them with glycated human serum albumin (G-HSA). We observed that G-HSA increased IL-17A secretion but also, Interferon gamma (IFNγ), and Tumor necrosis factor alpha (TNFα) secretion and that anti-RAGE mAb specifically inhibited IL-17A secretion.

MATERIALS AND METHODS

Isolation and expansion of ASC

Subcutaneous or visceral AT samples were isolated from residues of bariatric surgery of obese subjects (body mass index > 30 kg/m²), or visceral surgery of lean controls with the informed consent of patients. AT samples (50-100 mg) were fragmented and incubated in 2 g/L of collagenase type Ia solution (Sigma Aldrich, C2674) dissolved in Dulbecco's modified eagles medium:Ham F12 (DMEM:F-12) medium (1:1 mL/L) (Invitrogen) for 40 min at 37 °C by mixing. Collagenase action was quenched by the addition of 1:1 mL/L of DMEM:F-12 medium supplemented with 10% heat inactivated fetal calf serum (FCS). The released stromal vascular fraction (SVF) was recovered by centrifugation (800 g for 7 min at 25 °C). Residual red blood cells were lysed by hypotonic shock and the ASC component of SVF was selectively expanded in culture medium composed of DMEM:F-12 supplemented with 10% FCS, 2 mmol/L L-glutamine and 100 U/mL penicillin-streptomycin. Half of the culture medium was changed every two to 3 d. ASC were amplified by several passages in culture (3 to 4) and directly used for experiments or stored in liquid nitrogen. The multipotent phenotype of ASC was validated by differentiating ASC into AD or osteoblasts, depending on the differentiation medium used, as previously reported[25]. ASC phenotype was assessed by staining with fluorescein isothiocyanate (FITC)-conjugated, phycoerythrin (PE)-conjugated, allophycocyanin (APC)-conjugated mouse anti-human cell surface markers (from ImmunoTools GmbH, Friesoythe, Germany) as recommended by the International Society for Cellular Therapy[27], and revealed a cluster of differentiation (CD) 90+, CD105+, CD73+, and CD45- pattern (Supplement Figure 1).

Isolation of blood MNC

Blood samples were obtained through the Blood Bank Center of Lyon (France), following institutionally approved guidelines. MNC were harvested from healthy human peripheral blood by density gradient centrifugation (Ficoll-Histopaque Sigma-Aldrich, Saint-Quentin Fallavier, France). MNC were stored in liquid nitrogen prior to use.

Co-culture assays and blockade experiments

ASC were harvested and seeded in 96-well plates (20000 cells/well) for 18-24 h in 200 µL of basal culture medium (DMEM:F-12 medium, 1:1 mL/L supplemented with 10% FCS). 100000 MNC were co-seeded for 48 h in the presence or absence of phytohaemagglutinin (PHA), 5 µg/mL (Sigma-Aldrich). Different ratios of ASC:MNC were used, as indicated in figure legends. Cells were incubated in Roswell Park Memorial Institute medium 1640 supplemented with either 1% human serum albumin (HSA) or 1% G-HSA, both from Sigma Aldrich (Saint Quentin-Fallavier, France). Supernatant was harvested after 48 h, and frozen. In blockade experiments anti-RAGE monoclonal antibody (RetD Systems, Lille, France) was added at 20 µg/mL during the whole period of culture.

Flow cytometry

FITC, PE, or APC conjugated mouse anti-human CD73, CD90, CD105, CD3, CD41 CD62P, human leukocyte antigen (HLA)-DR, intercellular adhesion molecule 1 (ICAM-1), CD8 (all from Immunotools) were used to label the various cells tested. Analyses were performed using the "LSR II 3 lasers" cytofluorometer and the Diva software (both were from BD Biosciences).

Cytokine secretion

IL-17A, IL-1β, IL-6 and TNFα secretions were measured by ELISA, using the corresponding antibodies (e-Biosciences, Paris, France).

Statistical analysis

One- or two-way repeated measures ANOVA, were used to compare multiple criteria. When some values were missing, mixed effects analyses were used. When the ANOVA or mixed effects analyses were significant, Bonferroni post hoc tests were used to do two-by-two analyses, taking into account the multiple comparisons. Paired *t* tests were used to compare two criteria, in univariate analysis. Differences were considered as statistically significant when P value was < 0.05. The analyses were done using Graphpad Prism 8 software.

RESULTS

G-HSA only weakly increases the levels of IL-17A promoted by obese ASC

We have previously reported that obese ASC activate IL-17A production by T cells in the presence of PHA. To investigate whether glycated albumin would increase the levels of IL-17A, we co-cultured the cells either in the presence of 1% HSA, or 1% G-HSA. Graded concentrations of ASC were co-cultured with the optimal concentration of MNC and activated with PHA. Although IL-17A secretion weakly increased, the two-way ANOVA multi-comparison tests did not show significant results whether HSA or G-HSA were added to cultures. But TNFα clearly increased ($P = 0.0165$ in two-way ANOVA). Thus, these results demonstrated a weak, but non-significant effect of G-HSA on Th17 stimulation by obese ASC, but an increase in TNFα production.

Lean ASC mediate higher levels of IL-17A, TNFα, and IFNγ secretion by T cells, in the presence of G-HSA

Because we have previously reported that lean ASC mediate IL-17A production at much lower levels than obese ASC, we investigated whether AGE could increase this production. Therefore, we co-cultured lean ASC with MNC in the presence of HSA, or G-HSA, and activated the co-cultures with PHA. Secretion of IL-17A was measured and showed a significant increase in the presence of G-HSA ($P = 0.0196$ in post-hoc Bonferroni tests). Interestingly, T helper 1 cytokines were also increased in the presence of G-HSA such as IFNγ ($P = 0.0065$ in Bonferroni post-hoc tests), and TNFα ($P = 0.0037$ in Bonferroni post-hoc tests). However, IL-6 and IL-1β, which are mostly

secreted by ASC and monocytes in this model, did not show significant differences in post-hoc Bonferroni tests, even though mixed effect analyses showed significancy, suggesting a specific effect of G-HSA on T cells.

G-HSA increases RAGE and HLA-DR expression in ASC/MNC co-cultured cells

We then investigated whether RAGE expression would be increased in the co-cultures of lean ASC and T cells leading to IL-17A production. We observed that the expression of RAGE was clearly increased when G-HSA was present. Moreover, HLA-DR expression was upregulated together with RAGE expression, in the presence of G-HSA.

Previous reports have demonstrated that glycated albumin induces platelet aggregation and activation[28,29]. Therefore, we measured the expression of CD62P and CD41 surface molecules, which are markers of platelet activation and aggregation, respectively, in experiments where T cells were either cultured alone, or co-cultured with ASC, in the presence of PHA and G-HSA, or HSA. Whereas markers of platelet aggregation and activation increased in activated ASC/MNC co-cultures, no difference was observed whether G-HSA or HSA was present. ICAM-1 expression, which has also been shown to increase in endothelial cells under the influence of RAGE activation[30] and in co-cultures of obese ASC with T cells[31], did not increase in the presence of G-HSA.

Therefore, these results suggested a specific effect of G-HSA on RAGE and HLA-DR expression in co-cultured cells.

Anti-RAGE mAb inhibits RAGE and HLA-DR expression in co-cultured cells

To better define the effects of G-HSA on RAGE and HLA-DR expression, we then added anti-RAGE mAb during co-cultures of lean ASC with MNC for 48 h, and measured the expression of RAGE, HLA-DR, CD41, CD62P and ICAM-1. As expected, RAGE expression decreased. Among the other molecules that were analyzed, only HLA-DR expression decreased down to the levels of cells co-cultured in the presence of HSA.

Specific inhibitory effects of anti-RAGE mAb on ASC-mediated IL-17A production

Because the anti-RAGE antibody was able to inhibit RAGE and HLA-DR expression, we then investigated whether anti-RAGE mAb could inhibit IL-17A production. Therefore, co-cultures of PHA-activated ASC/MNC cells were performed in the presence or absence of anti-RAGE mAb. Results showed that IL-17A secretion significantly decreased in the presence of anti-RAGE mAb ($P = 0.0402$ in paired t tests), but not IFNγ, nor TNFα, although a trend was observed for the latter. Therefore, our results suggested that RAGE might be specifically implicated in lean ASC-mediated IL-17A production, but not in IFNγ or TNFα secretion.

DISCUSSION

IL-17A/F are pro-inflammatory cytokines known to play an important role in AT-low grade inflammation in obese individuals, possibly leading to T2D[25,32-36]. Interestingly, IL-17A/F cytokines have also been involved in the pathogenicity of T1D[37], notably through their peri-pancreatic fat location[38,39]. Indeed, deletion of sentrin-specific protease 1 (SENP1), a SUMO-specific protease in AT, resulted in activating NFKB and pro-inflammatory cytokine/chemokine secretion in peri-pancreatic AT, ultimately leading to the recruitment of immune cells, including Th17 cells[38]. Subsequent to induced beta cell death and pancreatic disruption, spontaneous development of T1D was further observed in these SENP1-invalidated mice[39]. Strengthening the potential role of pancreatic fat as a pathogenic factor leading to beta cell dysfunction is the demonstration that pancreatic fat has been negatively associated with insulin secretion in individuals with impaired fasting glucose and/or impaired glucose tolerance[40]. Moreover in this study, pancreatic fat was found to be a stronger determinant of impaired insulin secretion than visceral fat[40]. In the present study, we investigated whether AGE could be involved in the dysfunction of lean AT, through increase of IL-17A production by T cells interacting with adipocyte progenitors. To address this question, we used the co-culture model that we have previously reported to lead to Th17 cell activation by ASC[25], and added low concentrations of HSA or G-HSA. When using obese ASC, we observed only a weak, but not significant effect of G-HSA on increased IL-17A production, suggesting other mechanisms than AGE in obese-ASC-mediated IL-17A secretion (Figure 1). However, when lean ASC/MNC co-cultures

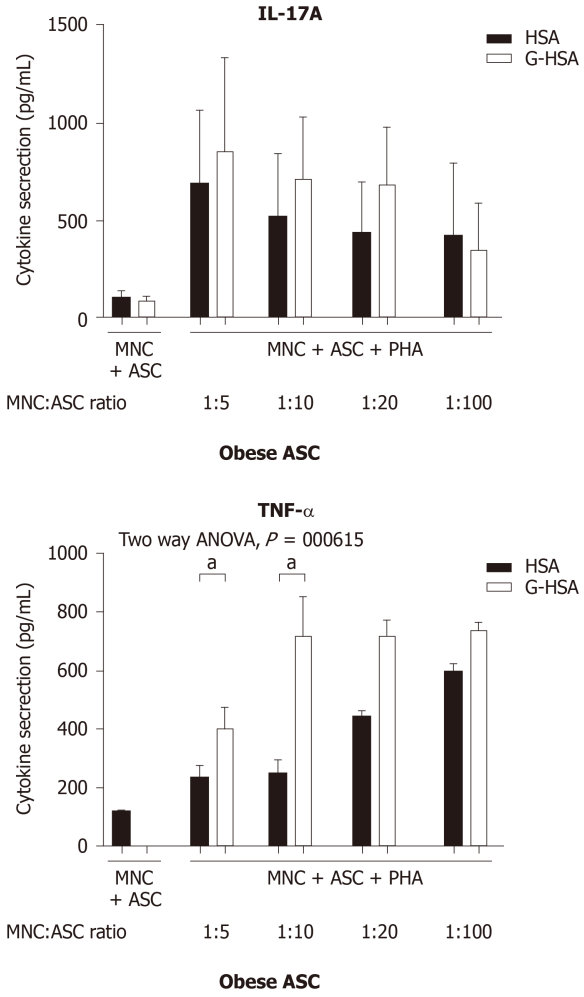

Figure 1 Glycated human serum albumin increases the levels of interleukin 17A promoted by obese adipose-derived stem cells, at suboptimal conditions. Graded concentrations of obese adipose-derived stem cells (ASC) were co-cultured in the presence of mononuclear cells (MNC) at different ratios of 1:5, 1:10, 1:20, or 1:100, with 20000 ASC for 100000 MNC. Co-cultures were activated by phytohemagglutinin A (5 μg/mL) for 48 h in the presence of 1% human serum albumin or 1% glycated human serum albumin. ELISA were performed to measure interleukin-17A production and tumor necrosis factor alpha. Bars represent the mean ± SE of 4 independent experiments performed in triplicates. The *P* value shown in the figure corresponds to ANOVA multivariate analysis results, and $^{a}P < 0.05$, as obtained by Bonferroni post-hoc tests. ASC: Adipose-derived stem cells; MNC: Mononuclear cells; PHA: Phytohemagglutinin A; HSA: Human serum albumin; G-HSA: Glycated human serum albumin; IL: Interleukin; TNF-α: Tumor necrosis factor alpha.

were incubated in the presence of 1% G-HSA, a significant increase of IL-17A production was observed, together with increased IFNγ and TNFα production. This increase was probably related to specific activation of T cells by G-HSA, as neither IL-1β nor IL-6 significantly increase (Figure 2).

RAGE is one of the AGE receptors and has been widely implicated in most of the pro-inflammatory mechanisms mediated by AGE and leading to chronic inflammation disorders. They are constitutively expressed in T cells from diabetic patients, and are known to activate the NFKB pathway leading to inflammatory cytokine production[15]. However, not all T cells are regulated by RAGE, as shown by Chen *et al*[20] who demonstrated a differential effect of RAGE blockade on splenic T cells but not on fully activated T cells in a transfer model of diabetes. Supporting these results, we also demonstrated herein that RAGE was involved in Th17 cell, but not Th1 cell activation, since only IL-17A secretion was inhibited by anti-RAGE mAb (Figure 5). A similar differential effect of RAGE on IL-17A and TNFα production was also observed in T1D, where RAGE positive T cells were found to express higher levels of IL-17A but not TNFα nor IFNγ, as compared with RAGE negative cells in the same patients. This demonstrated thus a potentiating effect of RAGE signaling pathway on IL-17A production[22]. Confirming the implication of RAGE in ASC-mediated T cell activation, we observed increased RAGE expression, together with HLA-DR expression when G-HSA was added to the co-cultures, and an abolition of this effect in the presence of RAGE mAb which concomitantly resulted in inhibition of IL-17A production (Figures 4 and 5). Finally, although glycated albumin has been shown to increase platelet

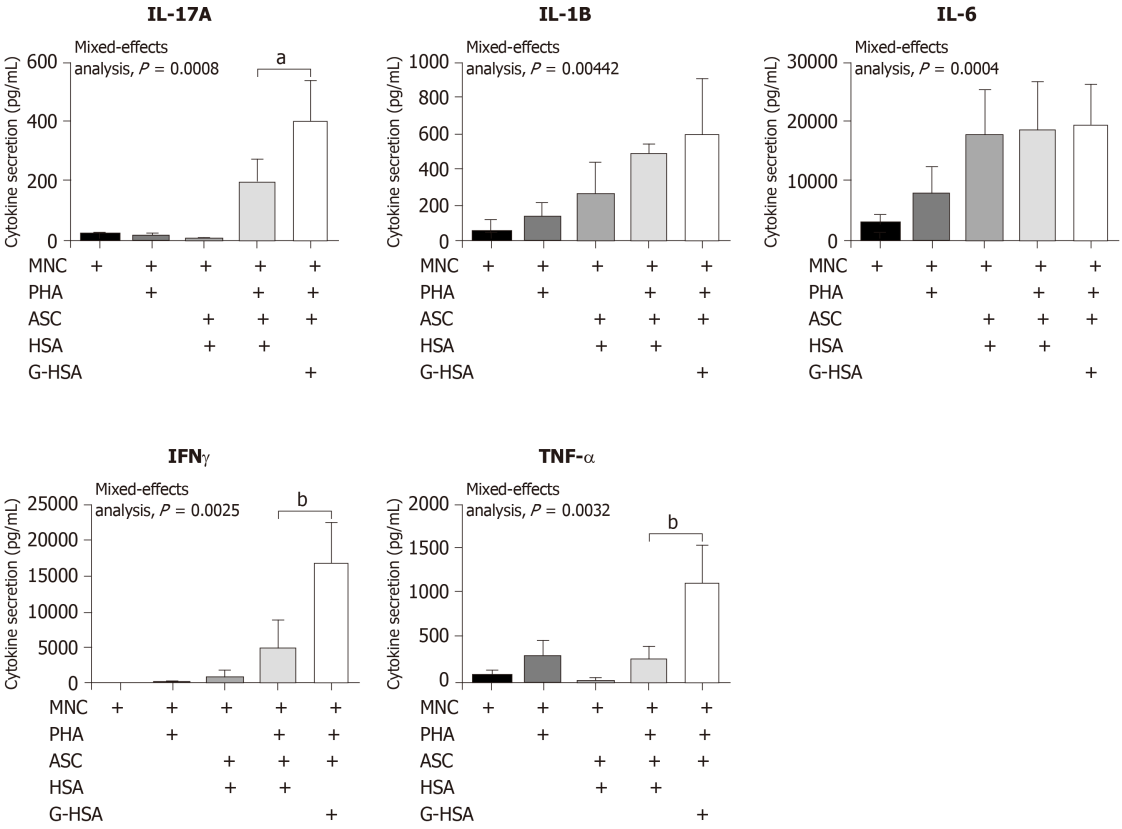

Figure 2 Lean adipose-derived stem cells increase the levels of interleukin 17A, tumor necrosis factor alpha, and interferon gamma secretion by mononuclear cells, in the presence of glycated human serum albumin. Lean adipose-derived stem cells (ASC) were co-cultured with mononuclear cells (MNC) at the 1:5 ASC to MNC cell ratio, in the presence of 1% glycated human serum albumin or human serum albumin (HSA) for 48 h, phytohemagglutinin A (PHA) was added or not at 5 μg/mL. As control, MNC were cultured alone in the presence of PHA or not, and HSA. ELISA were performed to measure interleukin (IL)-17A, IL-1β, IL-6, interferon gamma, and tumor necrosis factor alpha production. Bars represent the mean ± SE of 5 independent experiments performed in triplicates. The *P* values shown in the figure correspond to ANOVA multivariate results and $^{a}P < 0.05$, $^{b}P < 0.01$, respectively as obtained by Bonferroni post-hoc tests. ASC: Adipose-derived stem cells; MNC: Mononuclear cells; G-HSA: Glycated human serum albumin; HSA: Human serum albumin; PHA: Phytohemagglutinin A; IL: Interleukin; IFNγ: Interferon gamma; TNFα: Tumor necrosis factor alpha.

aggregation[28,29],we did not find its involvement in AGE-mediated activation of T cell secretion. Indeed, up-regulation of CD41 and CD62P expression, two markers of platelet aggregation and activation respectively, did not further increase in the presence of G-HSA (Figure 3). Moreover, RAGE mAb did not inhibit the expression of these two markers, either (Figure 4). ICAM-1 expression, which has been shown to be up-regulated by AGE in other cell models[41], did not increase in the presence of AGE, and was not inhibited by RAGE mAb (Figures 3 and 4). Therefore, we concluded that in our model platelet aggregation and ICAM-1 were not involved in the potentiation of Th17 cytokines production by G-HSA.

In conclusion, we have shown herein that the presence of G-HSA enhances lean ASC-mediated IL-17A production through a mechanism requiring RAGE signaling. Moreover, our study suggests a new mechanism by which ASC could contribute to inflammatory processes through AGE-mediated IL-17A production in AT of lean individuals. This could be potentially of importance in the context of T1D pathophysiology.

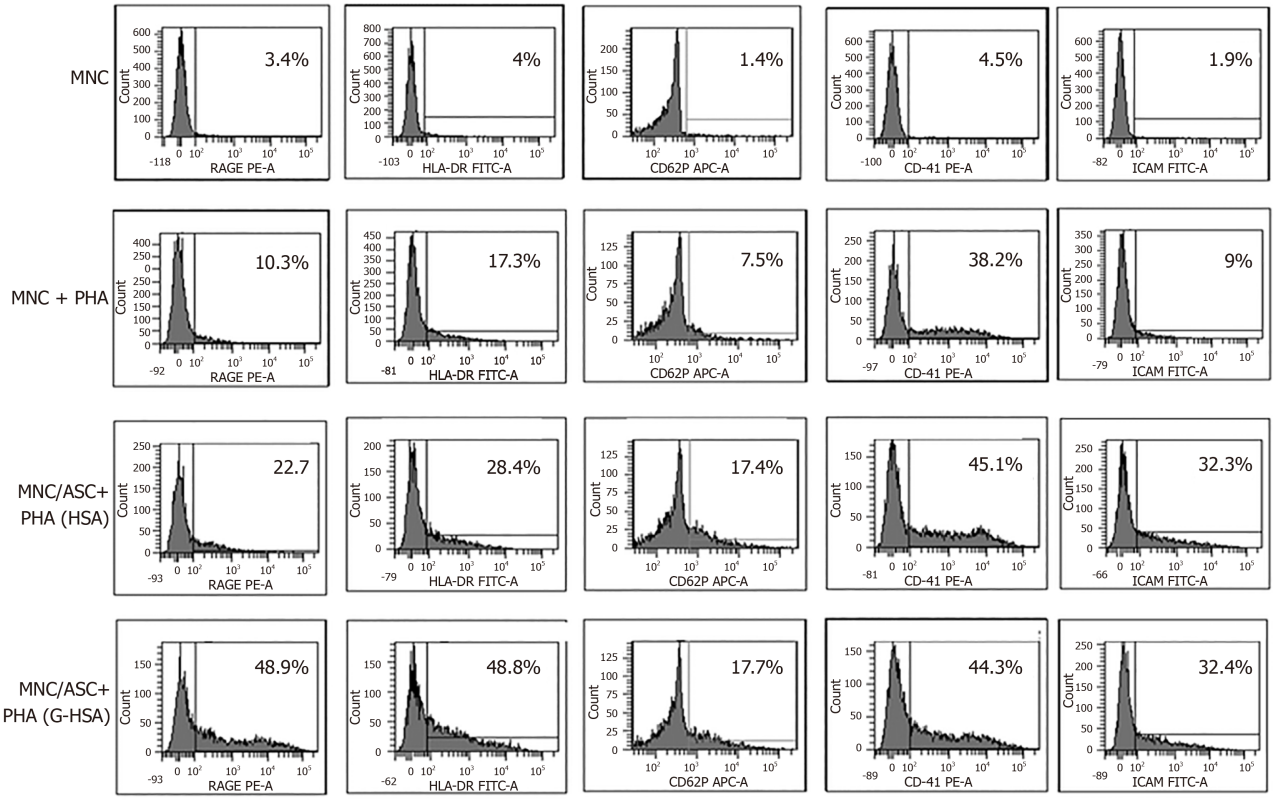

Figure 3 Glycated human serum albumin increases receptor for advanced glycated end products and human leukocyte antigen-DR expression in adipose-derived stem cell / mononuclear cell co-cultures. Lean adipose-derived stem cells (ASC) were co-cultured with mononuclear cells (MNC) at the 1:5 ASC to MNC cell ratio, in the presence of 1% glycated human serum albumin or human serum albumin (HSA) for 48 h, phytohemagglutinin A (PHA) was added or not. As control MNC were cultured alone in the presence or not of PHA, and HSA. Human leukocyte antigen-DR, receptor for advanced glycated end products, cluster of differentiation (CD) 41, CD62P, and intercellular adhesion molecule 1 were stained with fluorescent-conjugated antibodies, and analyzed by cytofluorometry, using the DIVA software. This experiment is representative of two experiments performed, with two different ASC. ASC: Adipose-derived stem cells; MNC: Mononuclear cells; G-HSA Glycated human serum albumin; HSA: Human serum albumin; PHA: Phytohemagglutinin A; HLA: Human leukocyte antigen; RAGE: Receptor for advanced glycated end products; CD: Cluster of differentiation; ICAM-1: Intercellular adhesion molecule 1; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; APC: Allophycocyanin.

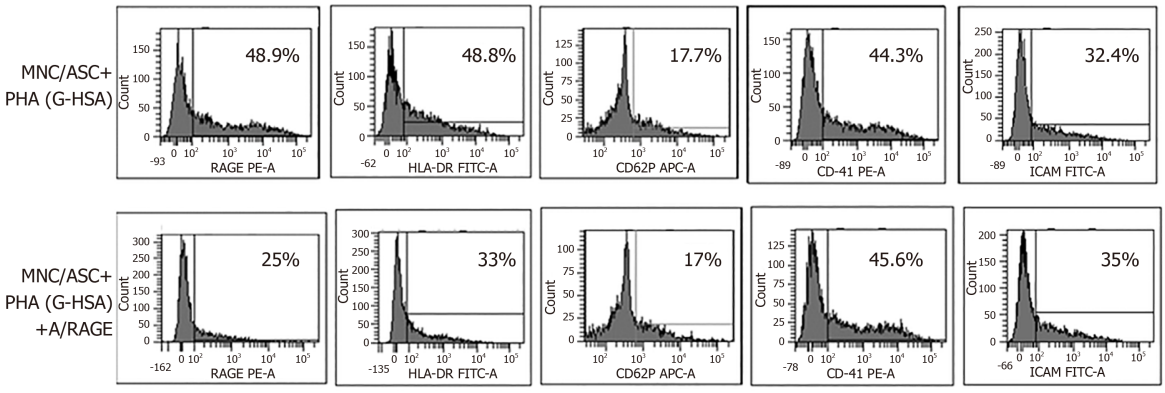

Figure 4 Anti- receptor for advanced glycated end products monoclonal antibody inhibits receptor for advanced glycated end products and human leukocyte antigen-DR expression. Lean adipose-derived stem cells (ASC) were co-cultured with mononuclear cells (MNC) at the 1:5 ASC to MNC cell ratio, in the presence of 1% glycated human serum albumin for 48 h, phytohemagglutinin A was added at 5 μg/mL. Anti-receptor for advanced glycated end products (RAGE) monoclonal antibody was added at a concentration of 20 μg/mL. cluster of differentiation (CD) 41, CD62P, intercellular adhesion molecule 1, human leukocyte antigen-DR, and RAGE expression were stained with fluorescent-conjugated antibodies and analyzed by cytofluorometry, using the DIVA software. This experiment is representative of two experiments performed with two different ASC. ASC: Adipose-derived stem cells; MNC: Mononuclear cells; G-HSA: Glycated human serum albumin; PHA: Phytohemagglutinin A; RAGE: Receptor for advanced glycated end products; mAb: Monoclonal antibody; CD: Cluster of differentiation; ICAM-1: Intercellular adhesion molecule 1; HLA: Human leukocyte antigen; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; APC: Allophycocyanin.

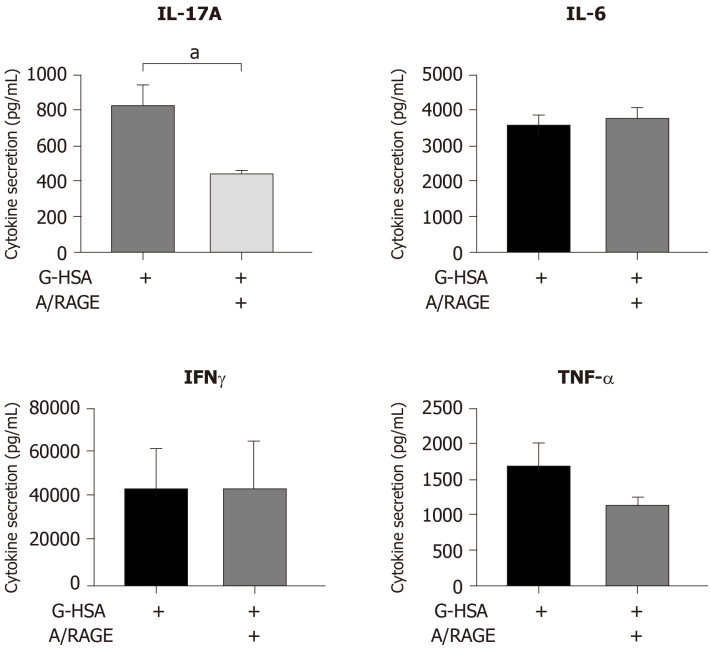

Figure 5 Inhibitory effects of anti-receptor for advanced glycated end products monoclonal antibody on interleukin 17A production. Lean adipose-derived stem cells (ASC) were co-cultured with mononuclear cells (MNC) at the 1:5 ASC to MNC cell ratio, in the presence of 1% glycated human serum albumin for 48 h, phytohemagglutinin A was added. Anti-receptor for advanced glycated end products monoclonal antibody was added at a concentration of 20 μg/mL. ELISA were performed to measure interleukin (IL)-17A, interferon gamma, tumor necrosis factor alpha and IL-6 secretion. Bars represent the mean ± SE of 5 independent experiments performed in triplicates. $^{a}P < 0.05$, as obtained by paired *t* tests. ASC: Adipose-derived stem cells; MNC: Mononuclear cells; G-HSA: Glycated human serum albumin; PHA: Phytohemagglutinin A; RAGE: Receptor for advanced glycated end products; mAb: Monoclonal antibody; IL: Interleukin; IFNγ: Interferon gamma; TNFα: Tumor necrosis factor alpha.

ARTICLE HIGHLIGHTS

Research background

Advanced glycation end products (AGE) are involved in type 1 diabetes (T1D) through reduction of glucose uptake and attenuation of insulin sensitivity. Moreover, AGE are known to promote interleukin (IL)-17A secreting T cells.

Research motivation

Adipose Tissue (AT), and especially pancreatic AT is a pathogenic factor leading to beta cell destruction partly due to IL-17A secreting T helper (Th17) cell recruitment; IL-17A/F are pro-inflammatory cytokines known to play an important role in AT-low grade inflammation and propagation of inflammation outside AT.

Research objectives

We have previously shown that adipose-derived stem cells (ASC) promote Th17 cells in obese AT, but not or less in lean AT. Here, we investigated whether AGE could improve lean ASC ability to promote IL-17A production by T cells.

Research methods

With this aim, we cocultured ASC from lean AT with mononuclear cells in the presence of glycated human serum albumin (G-HSA) or human serum albumin. We then analyzed the influence of AGE by blocking their ability to bind to receptor of advanced glycated end products (RAGE). IL-17A and other pro-inflammatory cytokine secretions were measured, together with surface expression of RAGE, and other relevant molecules.

Research results

We have demonstrated herein that G-HSA enhances IL-17A, interferon gamma and tumor necrosis factor alpha secretion by MNC in the presence of ASC harvested from lean individuals. This effect involves the RAGE/AGE axis as assessed by anti-RAGE blocking monoclonal antibodies (mAb) and is associated with increased expression of RAGE and human leukocyte antigen-DR molecules.

Research conclusions

Thus, our results demonstrate that G-HSA is able to improve lean ASC-mediated IL-17A production in AT, suggesting a new mechanism by which AGE could contribute to T1D pathophysiology.

Research perspectives

Here we propose a mechanism by which AT can lead to the recruitment of Th17 cells in lean individuals through activation of the AGE/RAGE axis. Because pancreatic fat has been involved in the pathogenicity of T1D, this model deserves to be validated in animal studies, in order to evaluate the efficacy of RAGE blocking mAb as a therapeutic tool.

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ORIGINAL ARTICLE

Basic Study

Bone marrow mesenchymal stem cells induce M2 microglia polarization through PDGF-AA/MANF signaling

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Abstract

BACKGROUND

Bone marrow mesenchymal stem cells (BMSCs) are capable of shifting the microglia/macrophages phenotype from M1 to M2, contributing to BMSCs-induced brain repair. However, the regulatory mechanism of BMSCs on microglia/macrophages after ischemic stroke is unclear. Recent evidence suggests that mesencephalic astrocyte–derived neurotrophic factor (MANF) and platelet-derived growth factor-AA (PDGF-AA)/MANF signaling regulate M1/M2 macrophage polarization.

AIM

To investigate whether and how MANF or PDGF-AA/MANF signaling influences BMSCs-mediated M2 polarization.

METHODS

We identified the secretion of MANF by BMSCs and developed transgenic BMSCs using a targeting small interfering RNA for knockdown of MANF expression. Using a rat middle cerebral artery occlusion (MCAO) model transplanted by BMSCs and BMSCs–microglia Transwell coculture system, the effect of BMSCs-induced downregulation of MANF expression on the phenotype of microglia/macrophages was tested by Western blot, quantitative reverse transcription-polymerase chain reaction, and immunofluorescence. Additionally, microglia were transfected with mimics of miR-30a*, which influenced expression of X-box binding protein (XBP) 1, a key transcription factor that synergized with activating transcription factor 6 (ATF6) to govern MANF expression. We examined the levels of miR-30a*, ATF6, XBP1, and MANF after PDGF-AA treatment in the activated microglia.

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RESULTS

Inhibition of MANF attenuated BMSCs-induced functional recovery and decreased M2 marker production, but increased M1 marker expression *in vivo* or *in vitro*. Furthermore, PDGF-AA treatment decreased miR-30a* expression, had no influence on the levels of ATF6, but enhanced expression of both XBP1 and MANF.

CONCLUSION

BMSCs-mediated MANF paracrine signaling, in particular the PDGF-AA/miR-30a*/XBP1/MANF pathway, synergistically mediates BMSCs-induced M2 polarization.

Key Words: Mesencephalic astrocyte–derived neurotrophic factor; Bone marrow mesenchymal stem cell; Microglia/macrophage polarization; Endoplasmic reticulum stress; Cerebral ischemia/reperfusion injury

Core tip: Induction of M2 microglia/macrophage polarization may contribute to the mechanisms underlying the neuroprotective effect of bone marrow mesenchymal stem cells (BMSCs) in treating stroke. This is one of the few known studies exploring the soluble mediators responsible for interactions between BMSCs and microglia/macrophages. We demonstrated that BMSCs-mediated MANF paracrine signaling, in particular the PDGF-AA/miR-30a*/XBP1/MANF pathway, was involved in BMSCs-induced M2 phenotype polarization. Therefore, this novel molecular mechanism of BMSCs-based immunomodulatory effect on microglia/macrophages may be a novel promising therapeutic strategy for treatment of ischemic stroke.

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INTRODUCTION

Ischemic stroke following cerebral artery occlusion is a major cause of chronic disability worldwide and effective therapy to improve functional recovery after stroke is not available[1]. Bone marrow mesenchymal stem cells (BMSCs) are well known as rare multipotent cells and are characterized as potent modulators of regeneration and immune responses. BMSCs transplantation may be an effective multitarget therapeutic strategy to facilitate functional recovery after ischemic stroke through pleiotropic mechanisms[2-4].

Classic (M1) or alternative (M2) activation has been mostly reported for macrophage responses during peripheral inflammation, and recently, microglia were found to have a similar activation process upon ischemic insult[5]. M1-like microglia/macrophages secrete proinflammatory cytokines, such as inducible nitric oxide synthase (iNOS), and cause tissue damage. In contrast, M2-like microglia/macrophages secrete anti-inflammatory cytokines, such as Arginase-1 (Arg-1), supporting neural repair[6,7]. Recently, several *in vitro*[8,9] and *in vivo*[10,11] studies have shown that BMSCs promote M2 polarization and neurogenesis and tissue repair, probably depending on the trophic and growth factors secreted by BMSCs. However, the precise mechanism underlying BMSCs-induced M2 polarization is not yet clear[12].

Mesencephalic astrocyte-derived neurotrophic factor (MANF), also named as arginine-rich, mutated in early stage tumor (ARMET), is a soluble protein induced by endoplasmic reticulum (ER) stress to protect against ER-stress-induced damage[13-17]. Treatment with MANF significantly reduces ischemic brain injury and improves behavior in stroke in rats[18-21]. ER stress triggers activation of unfolded protein response (UPR), which comprises two prosurvival pathways that are controlled by stress sensor proteins in the ER membrane: Activating transcription factor 6 (ATF6)

and inositol-requiring enzyme-1(IRE1)[22-24]. After activation, IRE1 converts to an active endonuclease that cleaves unspliced X-box binding protein-1 (XBP1u) mRNA into spliced XBP1 (XBP1s) mRNA, which encodes the transcriptionally active XBP1s protein[25]. ER stress-induced transcriptional upregulation of MANF is driven by an ER stress response element (ERSE)-II in the MANF promoter, and is recognized by ATF6 or XBP1s[26,27]. The XBP1 arm of the UPR is thought to play a critical role in enhancing the M2-like macrophage phenotype[28]. A recent study has shown that damaged retina secretes platelet-derived growth factor-AA (PDGF-AA) that signals to innate immune cells to produce MANF, which promotes macrophage M2 activation and tissue repair[29]. Based on these observations, it would be logical to propose that PDGF-AA, secreted abundantly from BMSCs[30], regulates XBP1 expression, which enhances expression of MANF, contributing to M2 phenotype.

MicroRNAs (miRNAs) are a new class of endogenous, small, 19-25-nucleotide noncoding RNAs that act as negative regulators of gene expression by inhibiting mRNA translation or promoting mRNA degradation[31]. As the first discovery of a miRNA that directly modulates a UPR effector, ER stress-inducible miR-30c-2* limits expression of XBP1, which is a potential target of the miR-30* family[32,33]. Therefore, we hypothesized that miR-30a* (recently designated as miR-30a-3p) might participate in the exquisite regulation of XBP1, and PDGF-AA might influence XBP1 *via* miR-30a*. Here, we aimed to explore whether BMSCs-mediated MANF paracrine signaling and the PDGF-AA/miR-30a*/XBP1/MANF axis can drive M2 polarization.

MATERIALS AND METHODS

Animals

Adult male Wistar rats, aged 6-7 wk and weighing 260-280 g, immature male Wistar rats, aged 4 wk and weighing 100-150 g, and neonatal Wistar rats were obtained from the Experimental Animal Center of the Second Clinical College of Harbin Medical University (Harbin, China) and housed in pathogen-free conditions. The rats were kept under standard conditions of temperature (25 ± 2°C) and lighting (12 h light/dark cycle). The Ethics Committee of the First Clinical College of Harbin Medical University approved this study (No. 2019006).

BMSCs isolation, culture, and identification

Rat BMSCs were isolated and cultured as described previously[4]. Bone marrow was obtained from the femurs and tibias taken from immature Wistar rats, followed by 5 min of centrifugation at 1500 rpm. The cell pellets were resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin liquid (Invitrogen). Cells were seeded at 10^6/mL in a 25-cm^2 tissue culture flask. After 3 d of culture with 5% CO_2 at 37°C, nonadherent hematopoietic cells were removed, and the medium was replaced. Half of the medium was replaced every 3-4 d, and adherent cells were allowed to reach 80% confluence before they were subcultured after 0.25% trypsin–EDTA digestion (Sigma–Aldrich, St. Louis, MO, United States). The third- and fourth-generation cells were used for further experiments. To verify the phenotype of the isolated BMSCs, the cells were incubated with fluorescence-conjugated antibodies, including CD90–fluorescein isothiocyanate (FITC), CD73–FITC, CD45–FITC, and CD11b–phycoerythrin (PE) (1:100; Sigma–Aldrich). An isotype-matched antibody was used as a control. Labeled cells were analyzed using flow cytometry (Thermo Fisher Scientific, Waltham, MA, United States).

Genetic engineering of BMSCs

Small interfering RNAs (siRNAs) targeting rat MANF (siMANF1, siMANF2, siMANF3, and negative control; NC) were designed and synthesized by Genechem Co. Ltd. (Shanghai, China). SiRNA sequences for transient knockdown of MANF are: 5'-CAGGCGACTGCGAAGTTTGTA-3' for siMANF1 sense, 5'-AATCGGTTGTGCTACTACA TT-3' for siMANF2 sense, and 5'-CACCATATCCCT GTGGAGAAG-3' for siMANF3 sense. BMSCs were seeded at 70%-80% confluence 12 h before transfection. BMSCs were divided into five groups: Nontransfected BMSCs (control), siMANF1-transfected BMSCs, siMANF2-transfected BMSCs, siMANF3-transfected BMSCs, and NC-transfected BMSCs (BMSCs-NC). Cells were transfected after seeding using Lipofectamine 2000 (Invitrogen). The final siRNA concentration was 50 nmol/L. After 48 h of transfection and antibiotics selection, cells were collected

for protein and RNA extraction.

BMSCs transplantation

A total of 170 male adult Wistar rats were used. Mortality rate was about 22% during and after surgery, and 38 rats were excluded from the middle cerebral artery occlusion (MCAO) model. Specifically, seven rats died from bleeding from the jugular vein, 23 after surgery, and eight after anesthesia. Overall, 132 rats were randomly divided into six groups (n = 22): Sham operation, cerebral ischemia/reperfusion (I/R) injury, cerebral I/R injury with phosphate-buffered saline (PBS) treatment, cerebral I/R injury with BMSCs treatment, cerebral I/R injury with BMSCs-NC treatment, and cerebral I/R injury with BMSCs/siMANF treatment. The selected BMSCs, as well as those with the best efficiency of siRNA transfection, were prepared in PBS at 10^5 cells/μL and injected into the right striatum with a coordinate of 3 mm lateral to the midline, 0.4 mm anterior to the bregma, and 5 mm deep at a rate of 1 μL/min. For a shorter time to take effect, 10 μL of the cultured cells and the same volume of PBS were injected at 24 h before cerebral I/R injury. The needle was retained in place for 5 min after injection. The brain samples were collected from each group after 24 h of reperfusion for further analysis. An overview of *in vivo* experimental protocol is presented in Figure 1A.

MCAO

Focal cerebral I/R injury was induced by MCAO as described previously[4]. Animals were anesthetized with 3% pentobarbital sodium by intraperitoneal injection (40 mg/kg). The nylon threads (L3400) were purchased from Jialing Biotechnology Co. Ltd. (Guangzhou, China). The right common carotid artery, external carotid artery, and internal carotid artery were clearly isolated through a cervical midline incision. The thread was introduced into the end of the external carotid artery, and then the distal end of the external carotid artery was cut. After that, the thread was inserted to the internal carotid artery until showing a slight resistance. This was to ensure that the thread has already blocked the origin of the middle cerebral artery. After 2 h of ischemia, the suture was withdrawn to allow reperfusion. Sham-operated animals were subjected to the same procedure, except that the artery was not occluded. After surgery, the rats were kept in a warm box heated with lamps until they woke up and then returned to their home cages. Cerebral I/R injury model was evaluated after revival from anesthesia using the Zea-Longa suture-occluded method[34]. Rats with a neurological deficit score of 2-3 were considered to have had successful surgery and were used for further experiments.

Neurological function test

Behavioral recovery was assessed by a blinded observer using the modified Neurological Severity Scores (mNSS) and Bederson's score on days 1, 3, and 7 after MCAO surgery. mNSS was graded as 0-18 scores (0, normal; 18, maximal deficit). One score point represented an inability to perform a test or lack of a tested reflex[35]. Bederson's score was scored according to the following criteria[36]: 0, rats extend both forelimbs straight with no observable deficits; 1, rats keep the one forelimb to the breast and extend the other forelimb straight; 2, rats show decreased resistance to lateral push in addition to behavior in score 1 without circling; 3, rats twist the upper half of their body in addition to behavior in score 2.

Evaluation of infarct volume

After 7 d of reperfusion, the rats were killed, and the brains were rapidly removed and frozen at -80°C for 15 min. The frozen brains were cut into coronal 2-mm-thick sections. The brain slices were incubated in a 2% solution of 2,3,5-triphenylterazolium chloride (Sigma–Aldrich) in normal saline for 30 min at 37°C and then transferred to a 4% paraformaldehyde solution for fixation. After staining, digital photographs were taken. Areas of the infarction in each slice were measured with Image J software (National Institute of Health, Bethesda, MD, United States). In order to minimize the error caused by brain edema, the relative infarct volume percentage was calculated as (volume of contralateral-normal volume of ipsilateral)/volume of contralateral × 100%[37].

Microglia isolation and culture

The preparation of mixed glial cell cultures and the isolation of microglia were carried out according to Saura *et al*[38]. The brains of neonatal (P1–P3) rats were isolated, then the meninges and choroid plexus were removed. The rat cortices were digested by 0.05% trypsin–EDTA for 15 min at 37°C. After centrifuging at 1200 rpm for 2 min, the

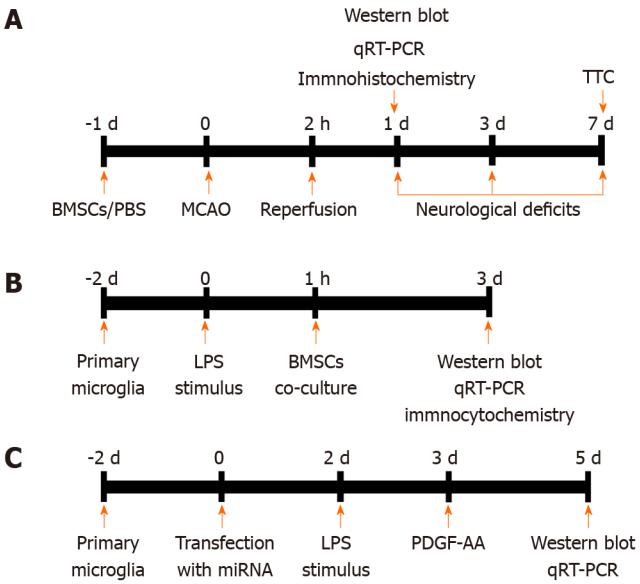


Figure 1 Experimental design. A: *In vivo* experiments. Bone marrow mesenchymal stem cells (BMSCs) or PBS were infused into the right striatum 1 d before MCAO. Animals were killed at 1 d for analysis by Western blot, qRT-PCR, and immunohistochemistry. Neurological function tests were evaluated at 1, 3, and 7 d post-stroke. Rats were killed at 7 d for TTC staining and measurement of infarct volume. B and C: *In vitro* experiments. Primary microglial cells were cultured for 2 d and exposed to 100 ng/mL LPS followed by indirect (Transwell) BMSCs coculture. After 2 d, cocultures were analyzed by Western blot, qRT-PCR, and immunocytochemistry (B). Cultured HAPI cells were transfected with miR-mimics, anti-miR, or corresponding negative control oligonucleotides for 24 h. PDGF-AA was added to the culture medium of HAPI cells pretreated with 100 ng/mL LPS for 24 h. After 2 d, cells were collected for Western blot and qRT-PCR (C). BMSCs: Bone marrow mesenchymal stem cells; PBS: Phosphate-buffered saline; MCAO: Middle cerebral artery occlusion; qRT-PCR: Real-time quantitative reverse transcription-polymerase chain reaction; TTC: 2,3,5-triphenylterazolium chloride; LPS: Lipopolysaccharide; PDGF-AA: Platelet-derived growth factor-AA.

cells were plated in 75-cm^2 flasks that had been coated with poly-L-lysine (Invitrogen). After 48 h, the nonadherent cells were removed by thorough washing of the surface with culture medium. Mixed glial cells were cultured in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine (Sigma–Aldrich), and 1% penicillin/streptomycin at 37°C in 5% CO_2 in air and 95% humidity for 2 wk. The culture medium was changed every 3 d. To harvest pure microglia, microglia on the confluent mixed glial cell layer were isolated by shaking the flasks for 2 h at 180 rpm. The medium containing the layer of detached microglia was collected and immediately centrifuged for 2 min at 1200 rpm. The supernatant was removed, and the obtained pure microglia were resuspended in fresh culture medium and seeded into subcultures. Microglial harvesting was repeated for a maximum of three times at intervals of 3 d. The purity of our primary microglia culture was assessed by immunocytochemical staining for ionized calcium-binding adapter molecule (Iba1).

Microglia polarization and coculture assay

Microglia were harvested from flasks and seeded at 10^6 cells/well on a 24-well plate with DMEM containing 10% FBS. After 24 h, the medium was replaced with 1 mL of FBS-free medium, and the cells continued to grow for 24 h. Microglia were unstimulated or stimulated with lipopolysaccharide (LPS) (Sigma–Aldrich) at 100 ng/mL for 24 h before coculture with BMSCs. To investigate the effect of spatial separation, we prepared Transwell experiments, where 10^5 BMSCs were plated into Transwell chambers with 1-μm-pore-sized membranes suitable for 24-well plates (Millipore, Temecula, CA, United States) and 10^6 microglial cells were added to the lower compartment. Untreated microglia served as a control, and conditioned medium of BMSCs in the upper chamber served as a vehicle. After coculture for 48 h, microglia were collected for further analysis. The study design is briefly illustrated in Figure 1B.

HAPI cell culture and transfection

Rat microglia cell line (HAPI) from the American-Type Culture Collection (ATCC) (Manassas, VA, United States) was cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO_2. The miR-30a* oligonucleotides (mimics, anti-miRNA, and corresponding NC) were prepared by Genechem. After 24 h of seeding HAPI cells in subculture (10^5 cells/well on a 24-well plate), transfection was

performed using 100 nmol/L Lipofectamine 2000 (Invitrogen). HAPI cells were divided into five groups: Nontransfected microglia (control), miR-30a*-NC-transfected microglia (miR-NC), miR-30a*-mimics-transfected microglia (miR-mimics), anti-miR-NC-transfected microglia (anti-NC), and miR-30a*-inhibitor-transfected microglia (anti-miR). After 24 h of transfection, gene and protein expression was determined.

Treatment of transfected HAPI cells

To determine whether the level of MANF is affected by various concentrations of PDGF-AA, the activated HAPI cells were exposed to recombinant rat PDGF-AA (R&D Systems, Minneapolis, MN, United States) at 0.1, 1, and 10 ng/mL for 48 h. Expression of MANF was detected by Western blot. To investigate the effect of PDGF-AA on LPS-stimulated microglia, the transfected HAPI cells were stimulated for 24 h using LPS (100 ng/mL). PDGF-AA at the selected concentration was added to the cell culture medium of HAPI cells for 48 h. The study design is briefly illustrated in Figure 1C.

Immunofluorescent staining

After anesthesia, transcardial perfusion with 0.1 mol/L PBS was performed, followed by perfusion with 4% paraformaldehyde (pH 7.4). The cerebral hemispheres were removed and placed in 4% paraformaldehyde for post-fixation for 24 h. After that, the brains were dehydrated with 30% sucrose in PBS until they sank to the bottom. Next, the brains were coronally sliced into 10-mm sections, which were fixed on slides and used for immunofluorescence staining, and blocked with 5% goat serum plus 0.1% Triton-X-100 at 20°C. The sections were incubated with primary antibodies as follows: Monoclonal mouse anti-Iba1 antibody (1:200, ab15690; Abcam, Cambridge, MA, United States); polyclonal rabbit anti-MANF antibody (1:500, PA5-20432; Thermo Fisher Scientific); polyclonal rabbit anti-iNOS antibody (1:200, ab15323; Abcam); and polyclonal rabbit anti-Arg1 antibody (1:200, ab91279; Abcam) overnight at 4°C. Sections were washed and incubated for 2 h at 20°C with secondary antibodies: Alexa-Fluor-488-conjugated goat anti-mouse IgG (1:200, ab150117; Abcam) and Alexa-Fluor-647-conjugated goat anti-rabbit IgG (1:200, ab150079; Abcam). The nuclei of cells were stained with 4'6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich). The proportion of positive cells was counted in five randomly selected fields from four sections of each brain sample, and the average was calculated. The images were taken under a fluorescent microscope (DP73; Olympus, Tokyo, Japan).

For immunocytochemistry analysis, cells were permeabilized for 15 min with 0.1% Triton X-100 in PBS and blocked for 1 h with PBS containing 0.1% Triton X-100, 2% bovine serum albumin, and 5% goat serum. Cells were incubated overnight with monoclonal mouse anti-Iba1 antibody (1:1000, ab15690; Abcam); polyclonal rabbit anti-MANF antibody (1:1000, PA5-20432; Thermo Fisher Scientific); polyclonal rabbit anti-iNOS antibody (1:1000, ab15323; Abcam); or polyclonal rabbit anti-Arg1 antibody (1:1000, ab91279; Abcam) at 4°C with gentle shaking, followed by 1-h incubation with Alexa-Fluor-488-conjugated goat anti-mouse IgG (1:1000, ab150117; Abcam) or Alexa-Fluor-488-conjugated goat anti-rabbit IgG (1:1000, ab150077; Abcam). Five pictures of each sample were taken by fluorescent microscopy, and the proportions of positive cells were counted.

Western blot analysis

Western blot was used to estimate expression of PDGF-AA/MANF and UPR-associated proteins. Rat brain tissues or cultured cells were homogenized in a commercially available buffer (RIPA Lysis Buffer, Strong; GenStar Biosolutions Co. Ltd., Beijing, China), with added dithiothreitol, phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Beyotime Biotechnology Co. Ltd., Shanghai, China). The homogenates were centrifuged at 14000 rpm for 15 min at 4°C. The supernatants were collected and protein concentrations were tested with a commercially available kit (BCA Protein Assay Kit; Beyotime Biotechnology). The samples were stored at -80°C until assay and were thawed only once. The cerebral samples or cell extracts were subjected to SDS-PAGE and transferred to polyvinyldifluoridine membranes (Millipore). The blocked membranes were incubated at 4°C overnight with primary antibodies. The primary antibodies were: Polyclonal rabbit anti-MANF antibody (1:500, PA5-20432; Thermo Fisher Scientific); polyclonal rabbit anti-PDGF-AA antibody (1:500, ab216619; Abcam); polyclonal rabbit anti-XBP1 antibody (1:1000, ab37152; Abcam); polyclonal rabbit anti-ATF6 antibody (1:1000, ab203119; Abcam); and monoclonal rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:1000, 5174; Cell Signaling Technology, Danvers, MA, United States). After rinsing, the membranes were incubated for 2 h at 20°C with horseradish-peroxidase-

conjugated anti-rabbit antibody (1:10000, 5151; Cell Signaling Technology). Protein was detected using the chemiDocXRS+ chemiluminescence imaging system (Bio-Rad, Hercules, CA, United States), and the density of the bands was determined with Image Lab image acquisition and analysis software (Bio-Rad).

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from rat brain tissues, BMSCs, and microglia by using TRIzol reagent (Invitrogen), and 5 μg of RNA was reverse transcribed with Revert Aid-M0MulV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania). A 20-μL reaction with Golden HS SYBR Green qPCR Mix (HaiGene, Harbin, China) was used to perform real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on Bio-Rad Min-Opticon2 (Bio-Rad). All primers used in this study were obtained from Invitrogen. Primer sequences are listed in Table 1. Thermal cycling conditions were 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Melt curves were performed upon completion of the cycles to ensure specificity of the product amplification. The expression of target mRNA was shown relative to the levels of GAPDH or U6, normalized to the control. The quantification of the genes of interest was calculated based on ΔΔCT and depicted as $2^{-\Delta\Delta CT}$.

Enzyme-linked immunosorbent assay

As a secretory neurotrophic factor, the secretion of MANF in culture medium of BMSCs was determined with an enzyme-linked immunosorbent assay (ELISA) kit (SAB, College Park, MD, United States). Standard curve and sample concentrations were calculated based on the mean of triplicate measurements for each sample.

Statistical analysis

Statistical analyses were performed with GraphPad Prism for Windows version 7 (San Diego, CA, United States), and the data are presented as the mean ± standard deviation (SD). All data were evaluated for normality by Kolmogorov–Smirnov test. The homogeneity of variance was confirmed using Bartlett's test before applying parametric tests to assess the null hypotheses ($P > 0.05$). Two-way analysis of variance (ANOVA) with Bonferroni *post hoc* test was performed for statistical analysis of mNSS and Bederson's score. The other comparisons between each group for statistical significance were performed by one-way ANOVA with Tukey's *post hoc* test for more than two groups. $P < 0.05$ was considered statistically significant.

RESULTS

In vitro construction of genetically modified BMSCs

The morphological features of the BMSCs were observed at passage 3 (Figure 2A). BMSCs were analyzed by flow cytometry, which showed that they were positive for cell surface antigens, CD90 and CD73, and negative for CD11b and CD45 (Figure 2B). We determined by ELISA whether BMSCs secrete MANF. After cell transfection and G418 (600 μg/mL) selection, qRT-PCR and Western blot showed that the BMSCs-siMANF2 group expressed the lowest MANF mRNA (Figure 2C) and protein (Figure 2D) levels in comparison with other groups. MANF secretion in the BMSCs-siMANF2 group was significantly decreased compared with that in the control and BMSCs-NC groups (Figure 2E). Thus, the BMSCs-siMANF2 group was used for further experiments. These results demonstrated that we have successfully developed the genetically modified BMSCs to downregulate MANF for subsequent cell transplantation.

Transplantation of BMSCs enhances functional outcomes and decreases infarction volume via production of MANF after 7 d in stroke rats

Similar to our previous experiments[39], intrastriatal BMSCs transplantation in I/R injury rats significantly improved neurological function compared to that in I/R and I/R + PBS groups. The mNSS and Bederson's score were increased in the I/R + BMSCs-siMANF group compared to those in the I/R + BMSCs and I/R + BMSCs-NC groups. However, both scores of the I/R + BMSCs-siMANF group were significantly lower than those of the I/R and I/R+PBS groups (Figure 3A and B). In line with the results of neurological function tests, TTC staining of rat brain slices further demonstrated that cerebral infarct volume of the I/R + BMSCs-siMANF group was significantly larger compared with that in the BMSCs and BMSCs-NC treated rats on

Table 1 Primers used for real-time quantitative reverse transcription polymerase chain reaction analysis

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
MANF	TCCGCTACTGTAAGCAAGGT	CTTCACCTAGGATCTTGGTG
PDGF-AA	GCCATTCCCGCAGTTTG	GGCTGGCACTTGACGCT
XBP1	ATGTTTTTCAAATGTCCTTCCCCAG	TGACAGAGAAAGGGAGGCTGGTAAG
ATF6	TGCAGGTGTATTACGCTTCGC	GCAGGTGATCCCTTCGAAATG
iNOS	ATCCCGAAACGCTACACTT	CGGCTGGACTTCTCACTC
Arg1	CAGTGGCGTTGACCTTGT	TGGTTCTGTTCGGTTTGC
NF-YA	CTGAGACTCCACAGCCATCA	GGATCTTCCCTTCTGCCTCT
NF-YB	CTGGATGGGGCTGACTGTAT	AGGAGGTACGCCAGTCTGTG
NF-YC	ACCCTTGCATGGCACTAAAG	GCTGGATGGTGGTCGTAGAA
*miR-30a**	CTTTCAGTCGGATGTTTGC	GTGCGTGTCGTGGAGTCG
GAPDH	GCAAGTTCAACGGCACAG	GCCAGTAGACTCCACGACAT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

MANF: Mesencephalic astrocyte–derived neurotrophic factor; *PDGF-AA*: Platelet-derived growth factor-AA; *XBP1*: X-box binding protein 1; *ATF6*: Activating transcription factor 6; *iNOS*: Inducible nitric oxide synthase; *Arg-1*: Arginase-1; *NF-Y*: Nuclear factor-Y; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

day 7 after cerebral I/R injury (Figure 3D). BMSCs treatment enhanced functional recovery and ameliorated cerebral ischemic damage in the acute cerebral I/R injury period by secreting MANF.

MANF secreted by BMSCs induces MANF expression in ischemic brains

MANF was induced at the early stage of cerebral ischemia. Infusion of BMSCs significantly increased MANF levels in the I/R and I/R + PBS groups at 24 h after MCAO. MANF protein and mRNA levels in the I/R + BMSCs-siMANF group were significantly decreased in comparison with those in the I/R + BMSCs and I/R + BMSCs-NC groups, but BMSCs-siMANF treatment totally attenuated the increased MANF levels in the ischemic brains (Figure 4A and B). It is known that MANF is expressed in microglia/macrophages and other central nervous system cells or infiltrating immune cells. The results of Western blot and qRT-PCR therefore reflected the changes in MANF in brain tissues of mixed cell types. To test whether BMSC treatment affects MANF expression in microglia/macrophages, we also detected MANF in the cells using immunofluorescent double staining with anti-MANF and anti-Iba1, a marker of microglia/macrophages. Unlike low expression of $MANF^+/Iba1^+$ in the sham group, increased numbers of $MANF^+/Iba1^+$ cells were detected in the I/R and I/R + PBS groups, suggesting that ER stress was involved in ischemia-induced activation of microglia/macrophages. BMSC treatment further elevated expression of $MANF^+/Iba1^+$ cells compared with that in the I/R and I/R + PBS groups. Consistent with Western blot and qRT-PCR results, there were significantly fewer $MANF^+/Iba1^+$ cells in the I/R + BMSCs-siMANF group than in the I/R + BMSCs and I/R + BMSCs-NC groups (Figure 4C). These results suggested that the beneficial effects of BMSCs might not be restricted to paracrine actions but might be due to their induction of host microglia/macrophages to produce MANF.

BMSCs-mediated MANF paracrine signaling upregulates MANF expression in microglia

The purity of our primary microglia culture was assessed by immunocytochemical staining with Iba1, being expressed by > 98% of the cells (Figure 5A). To test whether BMSCs affects expression of MANF in microglia, Transwell cocultures of microglia with BMSCs were established. LPS treatment significantly increased protein and mRNA levels of MANF in microglia, and these were significantly enhanced after coculture with BMSCs. In the LPS + BMSCs-siMANF group, BMSCs-induced MANF expression was significantly reduced, but not completely eliminated (Figure 5B and C). Accordingly, immunofluorescence showed that more $MANF^+$ microglia were found in the LPS and LPS + vehicle groups compared with the control group. Additionally, the

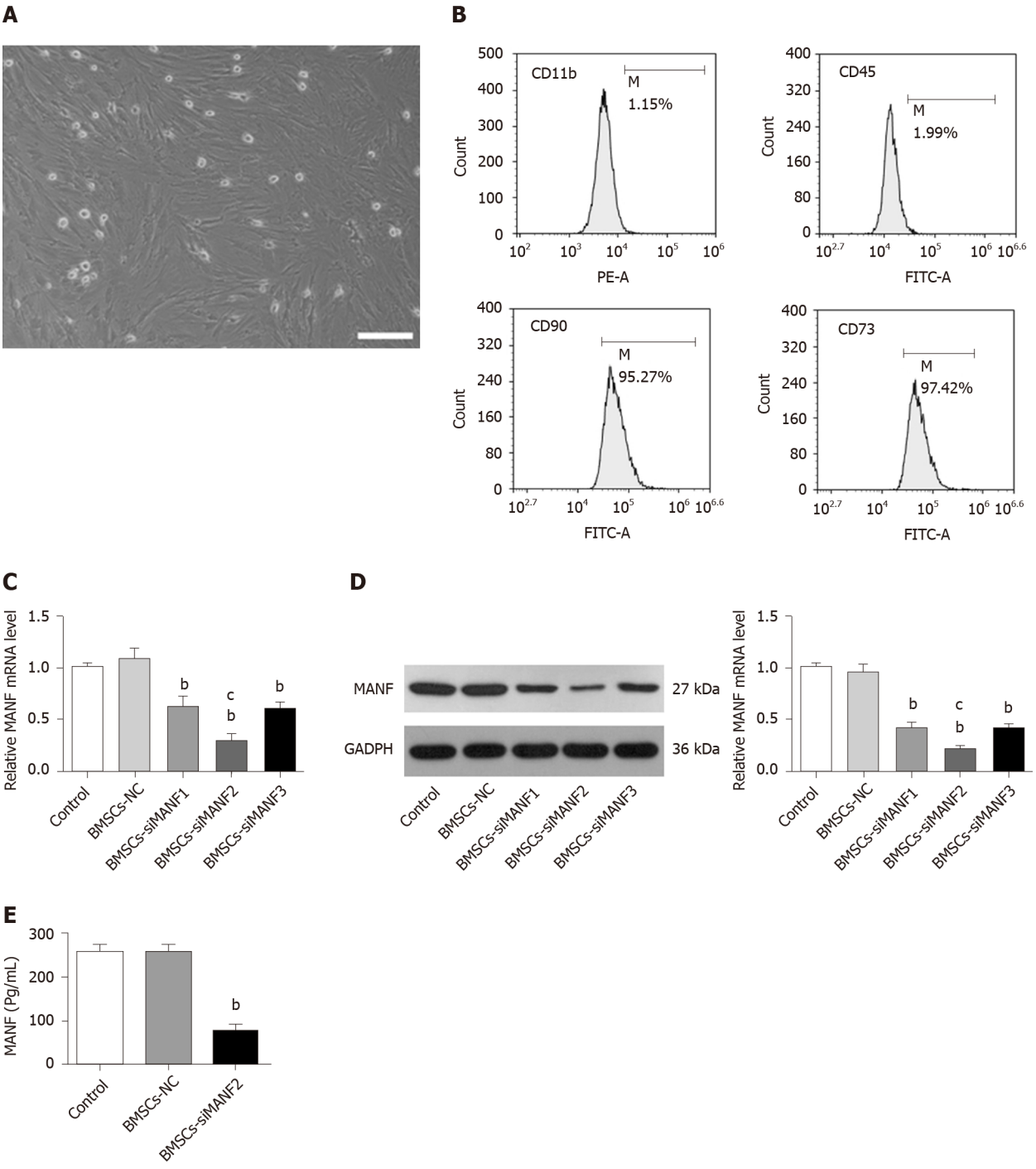


Figure 2 Construction of genetically modified bone marrow mesenchymal stem cells. A: Cultured bone marrow mesenchymal stem cells (BMSCs) were homogeneous in size and morphology. Scale bar = 500 μm; B: Flow cytometry for detection of BMSCs surface markers; C and D: qRT-PCR (C) and Western blot analysis (D) of MANF levels in BMSCs transfected with MANF-siRNA or NC. E: ELISA of MANF in culture medium of BMSCs. $^{b}P < 0.01$ *vs* Control and BMSCs-NC groups; $^{c}P < 0.05$ *vs* BMSCs-siMNAF1 and BMSCs-siMANF3 groups. The values are expressed as the mean ± SD (n=6). MANF: Mesencephalic astrocyte–derived neurotrophic factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; BMSCs: Bone marrow mesenchymal stem cells; BMSCs-NC: Negative control-transfected BMSCs; BMSCs-siMANF: MANF siRNA-transfected BMSCs.

number of MANF^{+} microglia was further increased after coculture with BMSCs. However, in the LPS + BMSCs-siMANF group, there were significantly fewer MANF^{+} microglia than in the LPS + BMSCs and LPS + BMSCs-NC groups (Figure 5D). These results demonstrated that the expression of microglia-derived MANF was activated by MANF secreted from BMSCs. We then tested whether MANF secreted from BMSCs regulates the levels of XBP1 mRNA and miR-30a*. As a key transcription factor of MANF, XBP1 mRNA expression was markedly upregulated in the activated microglia, and BMSCs induced further enhancement of gene expression (Supplement Figure 1A). As a shown in Supplement Figure 1B, miR-30a* level was downregulated in LPS-

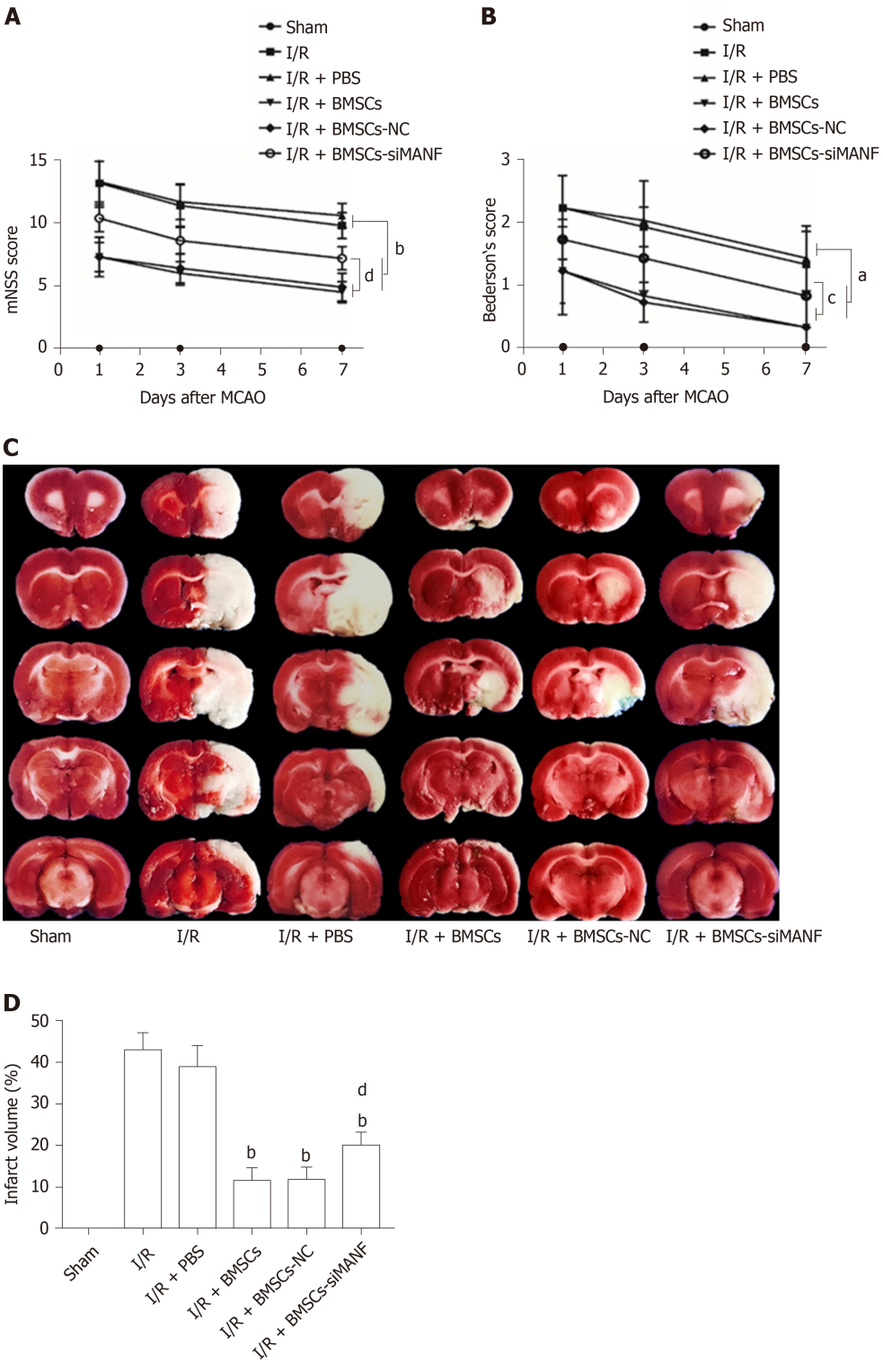


Figure 3 Effects of mesencephalic astrocyte–derived neurotrophic factor on post-stroke recovery and infarction volume in cerebral I/R rats treated with bone marrow mesenchymal stem cells. A and B: mNSS (A) and Bederson's (B) score evaluation of the behavior of rats on days 1, 3, and 7 after MCAO surgery; C and D: TTC staining (C) and quantitative analysis (D) of lesion volume on day 7 post-stroke. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* I/R and I/R + PBS groups; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* I/R + BMSCs and I/R + BMSCs-NC groups. The values are expressed as the mean ± SD (n =10). MANF: Mesencephalic astrocyte–derived neurotrophic factor; mNSS: Modified neurological severity scores; I/R: Ischemia/reperfusion; MCAO: Middle cerebral artery occlusion; PBS: Phosphate-buffered saline; BMSCs: Bone marrow mesenchymal stem cells; BMSCs-NC: Negative control-transfected BMSCs; BMSCs-siMANF: MANF siRNA-transfected BMSCs.

induced microglial cells, and further decreased after coculture with BMSCs. There was no difference in the levels of XBP1 mRNA and miR-30a* in the LPS + BMSCs-siMANF group compared to those in the LPS + BMSCs-NC and LPS + BMSCs groups. These results suggested that MANF did not affect the transcriptional levels of XBP1 and miR-30a*, and some soluble factors might regulate miR-30a*/XBP1 expression.

MANF secreted from BMSCs promotes M2 phenotype microglia/macrophages in ischemic brains

To test whether MANF plays an important role in BMSCs-induced M2 polarization, we evaluated the phenotypes of activated microglia/macrophages. Ischemia significantly increased the number of $iNOS^+/Iba1^+$ cells and iNOS mRNA expression at 24 h after I/R injury, while BMSCs significantly lowered both levels. iNOS expression in the I/R + BMSCs-siMANF group was significantly increased compared with that in the I/R + BMSCs and I/R + BMSCs-NC groups (Figure 6A and B). In contrast, ischemia increased the percentage of $Arg1^+/Iba1^+$ cells, and Arg1 mRNA levels were significantly enhanced by BMSCs infusion. The effect of BMSC-induced Arg1 expression was attenuated after BMSCs-siMANF transplantation (Figure 6C and D). The data indicated that the mechanism of BMSC-induced M2 polarization was related to the effect of secreted MANF on microglia/macrophages, leading to M2 phenotype after ischemic stroke.

MANF secreted from BMSCs induces microglia polarization to M2 phenotype

To identify the potential molecular mechanism underlying BMSC-induced M2 microglia, immunocytochemical staining and qRT-PCR were performed for iNOS and Arg1. Resting microglia did not express iNOS, and LPS upregulated iNOS expression. LPS-induced iNOS mRNA and protein expression was markedly decreased by coculture with BMSCs (Figure 7A and B). In contrast, untreated microglia expressed low levels of Arg1, and LPS stimulation reduced Arg1 expression in microglia. Expression of Arg1 was induced at mRNA and protein levels in LPS-stimulated microglia cocultured with BMSCs (Figure 7C and D). In addition, compared to coculture with BMSCs and BMSCs-NC, BMSCs-siMANF partially attenuated Arg1 or enhanced iNOS expression in microglial cells. Supporting the findings described *in vivo*, these results showed that BMSCs released MANF, thus resulting in the shift from M1 to M2 phenotype.

MiR-30a targets XBP1 in microglia*

XBP1 is a potential target of the miR-30* family, and overexpression of miR-30a* caused a significant decrease in XBP1 expression, with greater effect than the other miR-30* family members[33]. Therefore, to identify the roles for endogenous miR-30a* in XBP1 expression in microglia, HAPI cells were transfected with miR-mimics, miR-NC, anti-miR, or anti-NC. qRT-PCR and Western blot showed that cells transfected with miR-mimics showed a significant decrease in expression of XBP1, while inhibition of endogenous miR-30a* by synthetic anti-miR resulted in upregulation of XBP1 (Figure 8A and B). In contrast, MANF expression required ATF6 or XBP1. These transcription factors recognize ERSE and ERSE-II in the presence of the nuclear factor-Y (NF-Y, a heterotrimer of NF-YA, NF-YB, and NF-YC subunits)[40]. Therefore, to investigate whether miR-30a* targets other transcription factors besides XBP1, we performed qRT-PCR to analyze mRNA levels in HAPI cells. However, the ATF6 and NF-YA–C expression did not significantly change in any group (Figure 8C and D). These results suggested that miR-30a* inhibited expression of XBP1, targeting neither ATF6 nor NF-YA–C mRNA in microglia.

PDGF-AA causes XBP1 and MANF upregulation by inhibiting miR-30a expression*

We next sought to determine whether exogenous PDGF-AA attenuates expression of miR-30a* and thereby leads to an increased level of XBP1 and MANF. To assess the effect of PDGF-AA-induced MANF expression, various concentrations of PDGF-AA were evaluated. As shown by Western blot (Figure 9A), 0.1, 1, and 10 ng/mL of PDGF-AA significantly affected MANF expression in LPS-stimulated HAPI cells. Although PDGF-AA at lower concentrations of 0.1 and 1 ng/mL also significantly promoted MANF expression, 10 ng/mL of PDGF-AA revealed a greater effect. Thus, 10 ng/mL of PDGF-AA was used for further studies. There was a more significant decrease in miR-30a* expression in LPS-treated cells, and further downregulation after PDGF-AA treatment (Figure 9B). Transfection of miR-mimics reversed the suppressive effect of PDGF-AA on miR-30a* expression. As expected, ATF6, XBP1, and their downstream target MANF were significantly increased in LPS-induced ER stress processes of HAPI

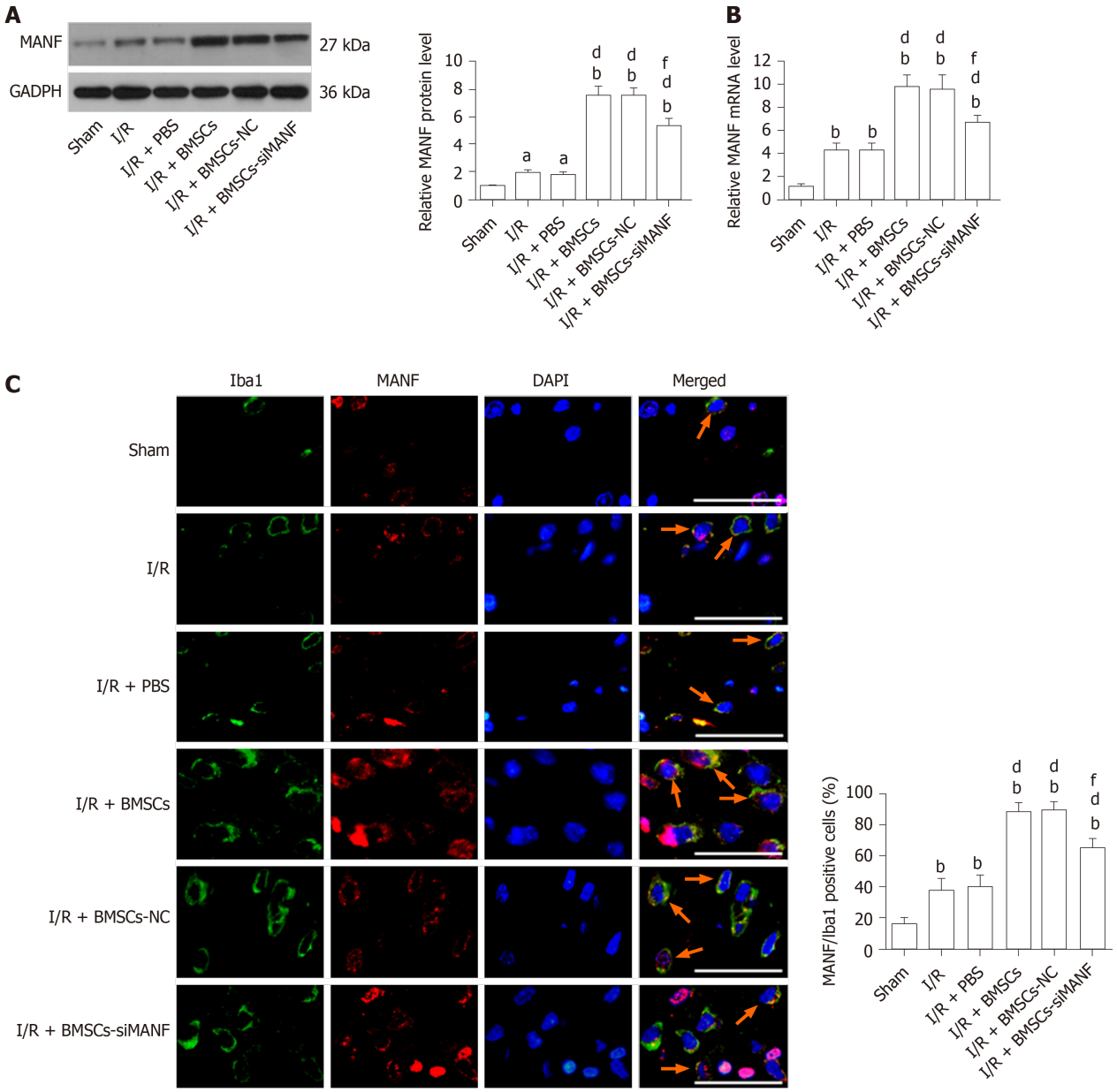


Figure 4 Mesencephalic astrocyte–derived neurotrophic factor expression in microglia/macrophages in cerebral ischemia. A and B: Western blot (A) and qRT-PCR analysis (B) of MANF expression at 24 h after cerebral I/R injury; C: Immunohistochemical analysis of MANF expression in the microglia/macrophages of the injured brains. Representative images of immunohistochemical staining for the microglia/macrophage marker Iba1 (green) and MANF (red), with DAPI (blue) as a nuclear counterstain, are shown. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Sham group; $^{d}P < 0.01$ *vs* I/R and I/R + PBS groups; $^{f}P < 0.01$ *vs* I/R + BMSCs and I/R + BMSCs-NC groups. Scale bar = 50 μm. Arrows point to the MANF^{+}/Iba1^{+} cells. The values are expressed as the mean ± SD ($n = 6$). MANF: Mesencephalic astrocyte–derived neurotrophic factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; I/R: Ischemia/reperfusion; PBS: Phosphate-buffered saline; BMSCs: Bone marrow mesenchymal stem cells; BMSCs-NC: Negative control-transfected BMSCs; BMSCs-siMANF: MANF siRNA-transfected BMSCs; Iba1: Ionized calcium-binding adapter molecule; DAPI: 4'6-diamidino-2-phenylindole.

cells. PDGF-AA treatment did not influence ATF6 expression (Figure 9C and D). In contrast, protein and mRNA expression of XBP1 and MANF was further induced by10 ng/mL of PDGF-AA. Overexpression of miR-30a* in HAPI cells attenuated induction of XBP1 (Figure 9E and F) and MANF (Figure 9G and H) in response to PDGF-AA, but it did not completely reduce XBP1 expression. These results suggested that the PDGF-AA/XBP1/MANF signaling pathway was, at least in part, mediated by miR-30a*.

MANF secreted by BMSCs does not influence PDGF-AA expression in ischemic brains or in microglia

We determined whether MANF paracrine signaling mediated by BMSCs affects expression of PDGF-AA *in vivo* and *in vitro*. We found that PDGF-AA expression was upregulated at 24 h after brain ischemia. Although BMSCs promoted PDGF-AA

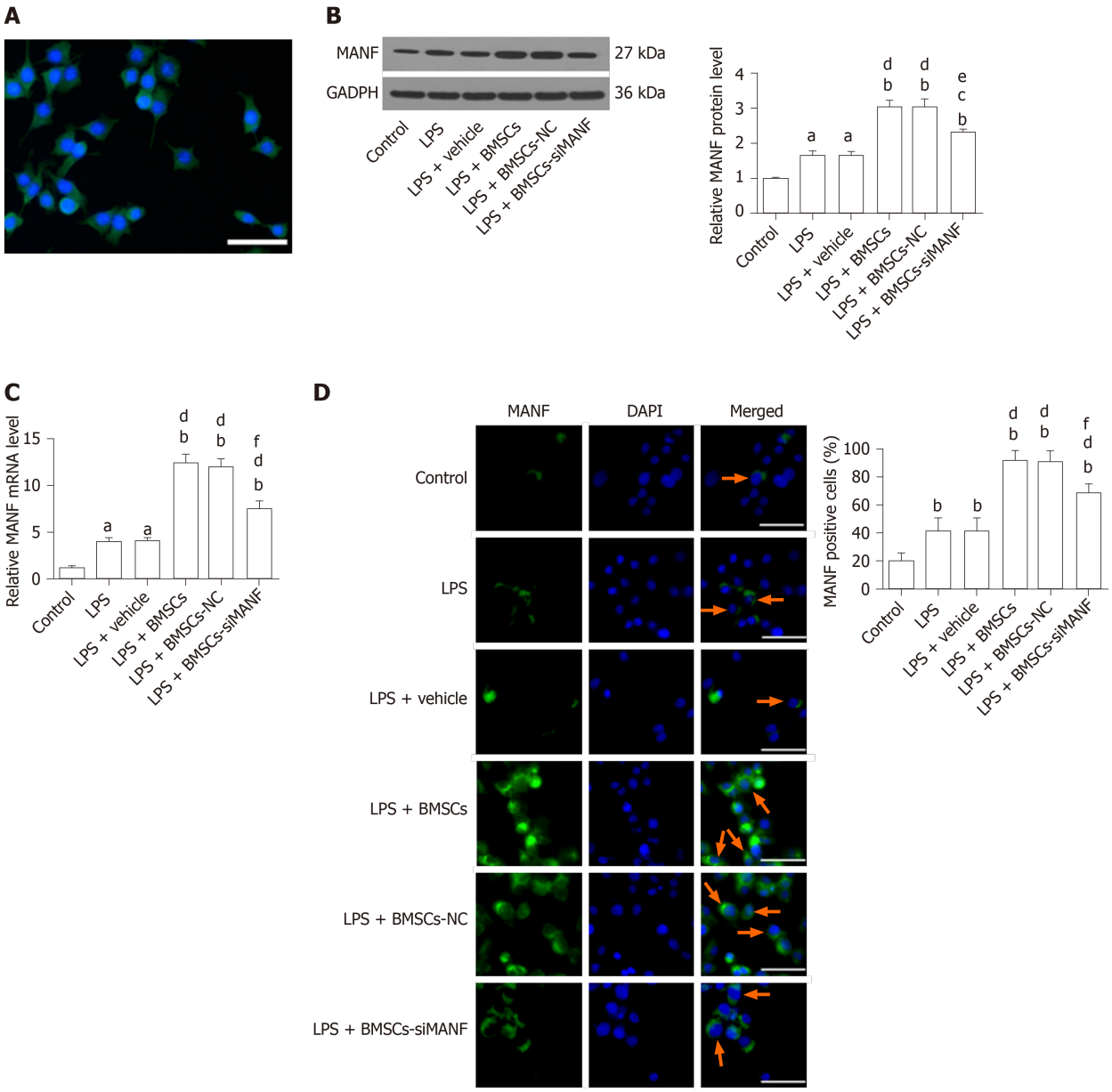
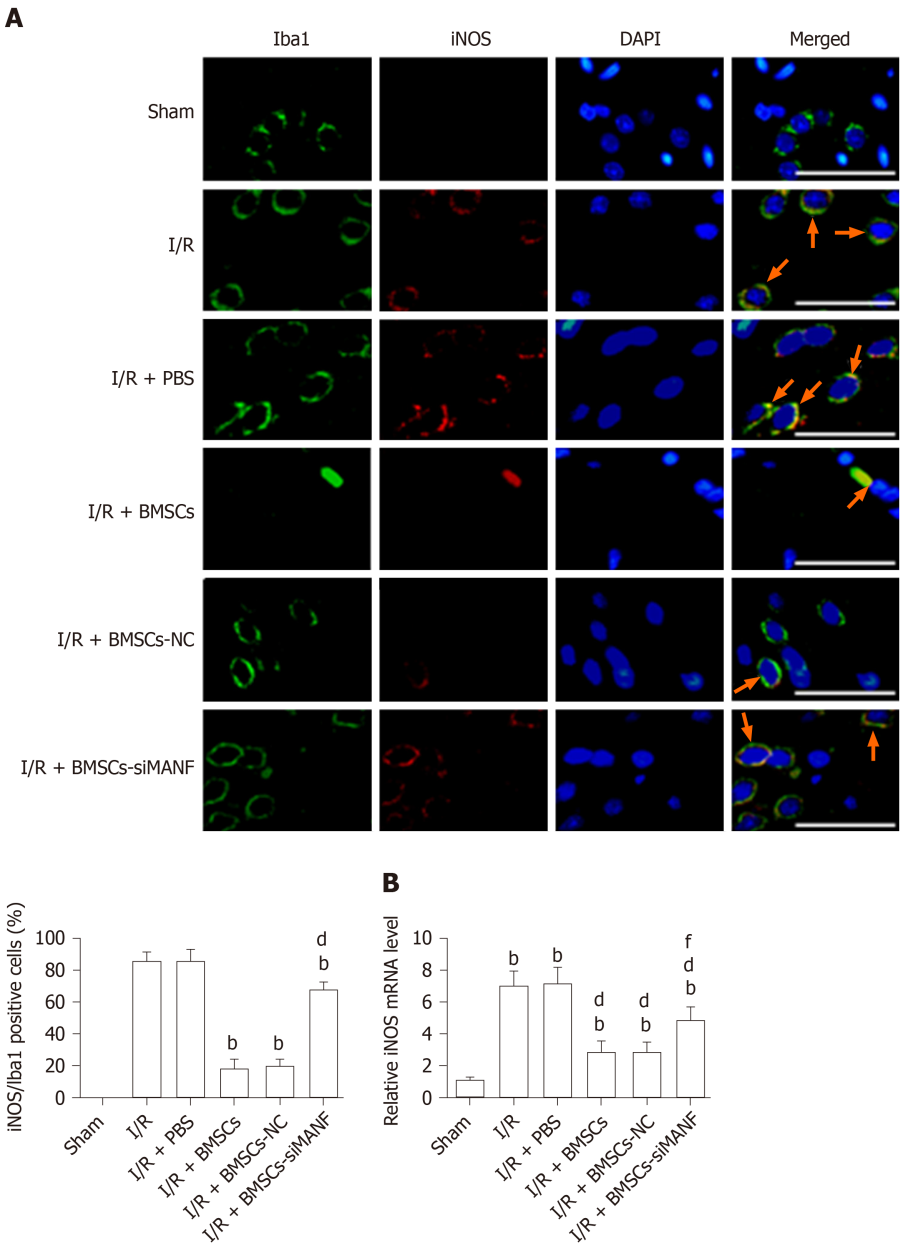


Figure 5 Mesencephalic astrocyte–derived neurotrophic factor expression in lipopolysaccharide-stimulated microglia. A: Representative immunocytochemical staining for Iba1 (green) and co-staining for DAPI (blue) as a nuclear counterstain. Scale bar = 50 μm; B–D: Western blot (B), qRT-PCR (C), and immunocytochemistry analysis (D) of MANF expression in microglia pretreated with 100 ng/mL LPS for 24 h with or without BMSCs. Representative images of MANF (green), with DAPI (blue) as a nuclear counterstain, are shown. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* LPS and LPS + vehicle groups; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* LPS + BMSCs and LPS + BMSCs-NC groups. Scale bar = 50 μm. Arrows point to the MANF^{+} cells. The values are expressed as the mean ± SD ($n = 6$). MANF: Mesencephalic astrocyte–derived neurotrophic factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; LPS: Lipopolysaccharide; BMSCs: Bone marrow mesenchymal stem cells; BMSCs-NC: Negative control-transfected BMSCs; BMSCs-siMANF: MANF siRNA-transfected BMSCs; DAPI: 4'6-diamidino-2-phenylindole.

expression, its protein and mRNA levels in the I/R + BMSCs-siMANF group did not show significant differences compared with those in the BMSCs and BMSCs-NC groups (Figure 10A and B). Similar to what we found *in vivo*, LPS-stimulated microglia produced a higher amount of PDGF-AA compared with the resting cells. Exposure to BMSCs significantly upregulated PDGF-AA expression in microglial cells, whereas the levels of PDGF-AA in the LPS + BMSCs-siMANF group were unchanged in comparison to those in the LPS+BMSCs-NC and LPS+BMSCs groups (Figure 10C and D). These findings allowed us to devise a new signaling pathway: BMSCs-secreted PDGF-AA and MANF synergistically increased MANF expression in the activated microglia. In addition, we provided evidence that PDGF-AA enhanced XBP1 expression *via* miR-30a* downregulation, resulting in MANF upregulation, finally leading to M2 polarization (Figure 10E).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Iba1
iNOS
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B
iNOS/Iba1 positive cells (%)
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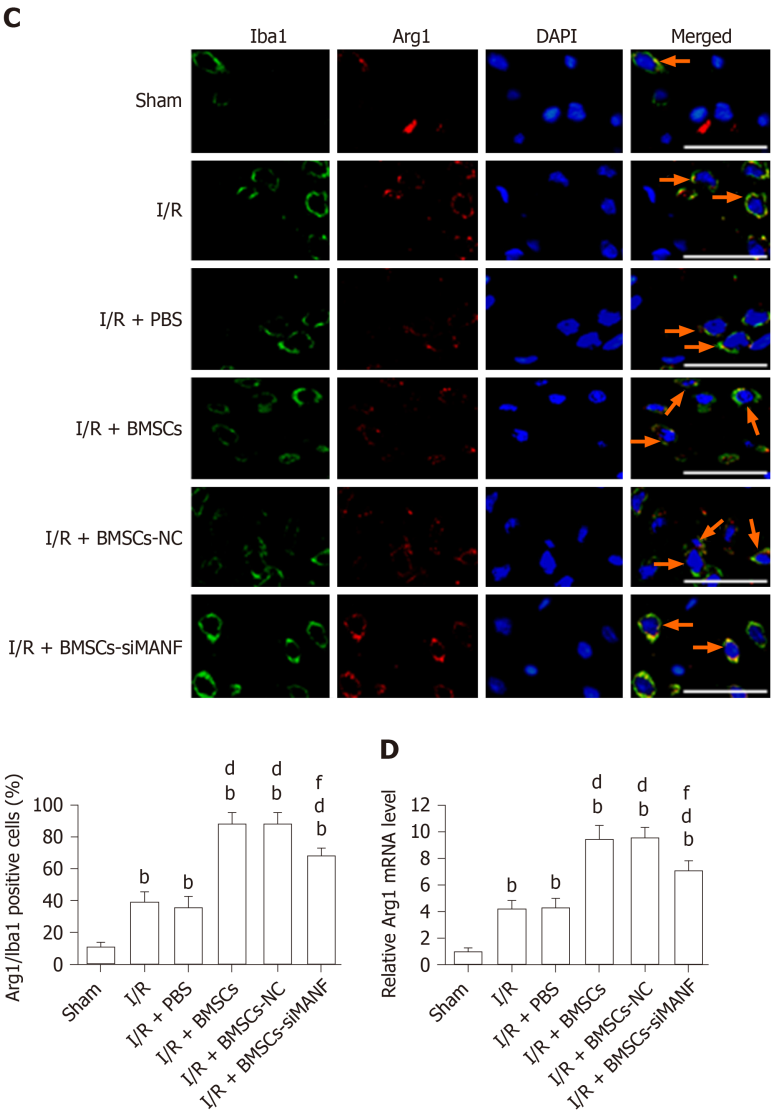
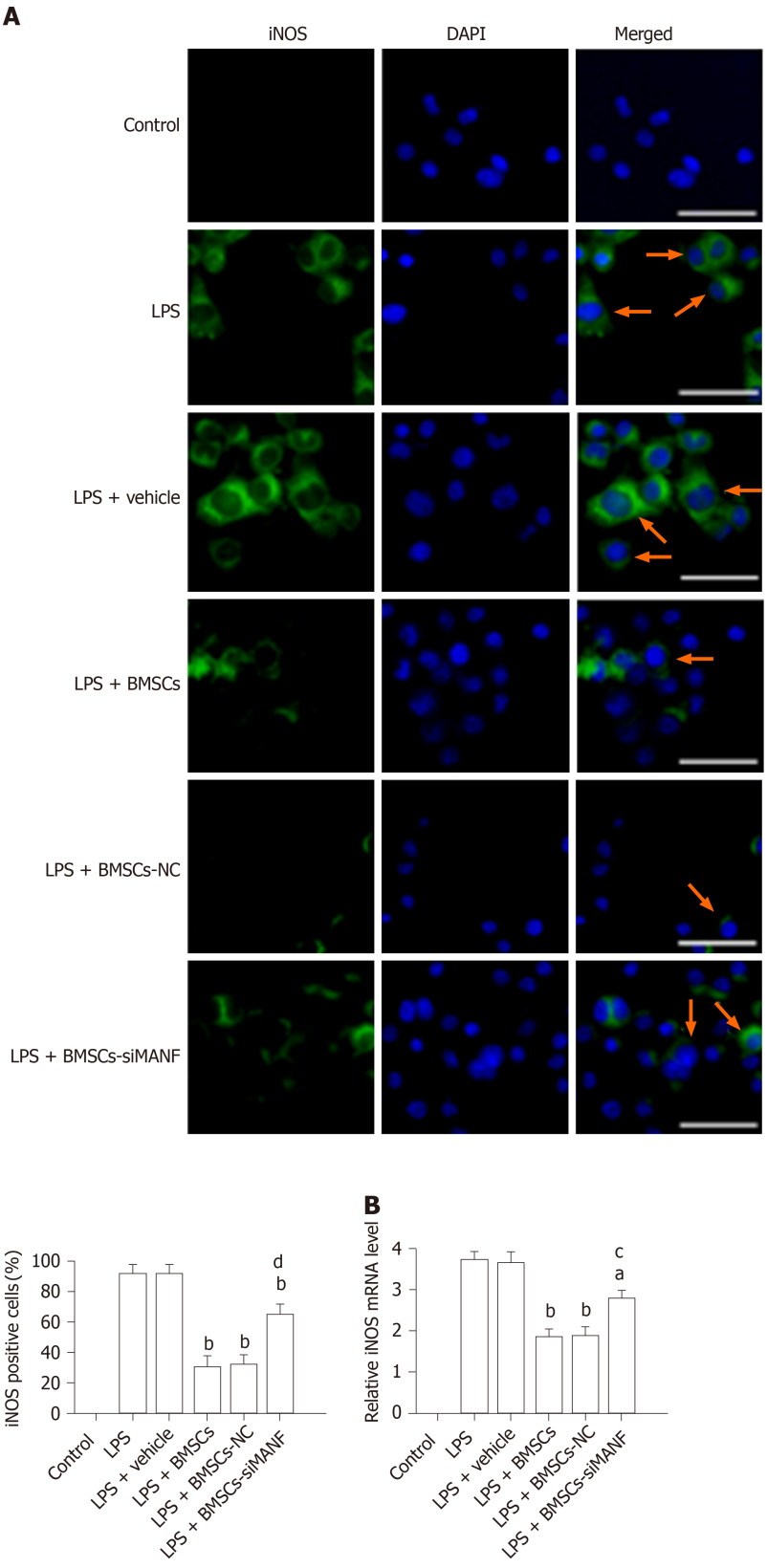


Figure 6 ***In vivo*** **analysis of M1 and M2 polarization markers after cerebral I/R injury with bone marrow mesenchymal stem cell treatment.**
A: Immunohistochemistry analysis of iNOS expression in the microglia/macrophages of the ischemic brain after 24 h of MCAO. Representative images of the indicated brain double stained with Iba1 (green) and iNOS (red), with DAPI (blue) as a nuclear counterstain, are shown. $^{b}P < 0.01$ *vs* I/R and I/R + PBS groups; $^{d}P < 0.01$ *vs* I/R + BMSCs and I/R + BMSCs-NC groups. Scale bar = 50 μm. Arrows point to the iNOS^{+}/Iba1^{+} cells; B: qRT-PCR analysis of iNOS expression in acute ischemic stroke rat brains; C: Immunohistochemical analysis of Arg1 expression in the microglia/macrophages of ischemic brains. Representative images of the indicated brain double stained with Iba1 (green) and Arg1 (red), with DAPI (blue) as a nuclear counterstain, are shown. Scale bar = 50 μm. Arrows point to the Arg1^{+} /Iba1^{+} cells; D: qRT-PCR analysis of Arg1 expression in rat brains at 24 h after MCAO. $^{b}P < 0.01$ *vs* Sham group; $^{d}P < 0.01$ *vs* I/R and I/R + PBS groups; $^{f}P < 0.01$ *vs* I/R + BMSCs and I/R + BMSCs-NC groups. The values are expressed as the mean ± SD ($n = 6$). I/R: Ischemia/reperfusion; PBS: Phosphate-buffered saline; BMSCs: Bone marrow mesenchymal stem cells; BMSCs-NC: Negative control-transfected BMSCs; BMSCs-siMANF: MANF siRNA-transfected BMSCs; iNOS: Inducible nitric oxide synthase; Arg-1: Arginase-1; Iba1: Ionized calcium-binding adapter molecule; DAPI: 4'6-diamidino-2-phenylindole.

DISCUSSION

In this study, we reported for the first time the contribution of MANF in BMSCs-induced M2 phenotype polarization as well as in the functional outcomes after stroke. Importantly, the *in vitro* study confirmed that BMSCs drove M2 microglia polarization through MANF secretion. We also studied the mechanisms underlying the PDGF-AA/MANF signaling pathway and found that PDGF-AA enhanced XBP1 expression by miR-30a* downregulation, leading to increased MANF expression in response to ER stress in the activated microglia.

Ischemic injury to the brain involves activation and mobilization of microglia and recruited macrophages, followed by coordinated balance of M1 and M2 phenotypes. M2-like microglia/macrophages can suppress inflammation and promote neuronal network recovery through tissue remodeling by growth factors, cytokines, and proteinases[41]. Inflammatory and regenerative responses are tightly co-regulated during tissue repair, and the proinflammatory microenvironments negatively affect


A
iNOS
DAPI
Merged
Control
LPS
LPS + vehicle
LPS + BMSCs
LPS + BMSCs-NC
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LPS + BMSCs-siMANF

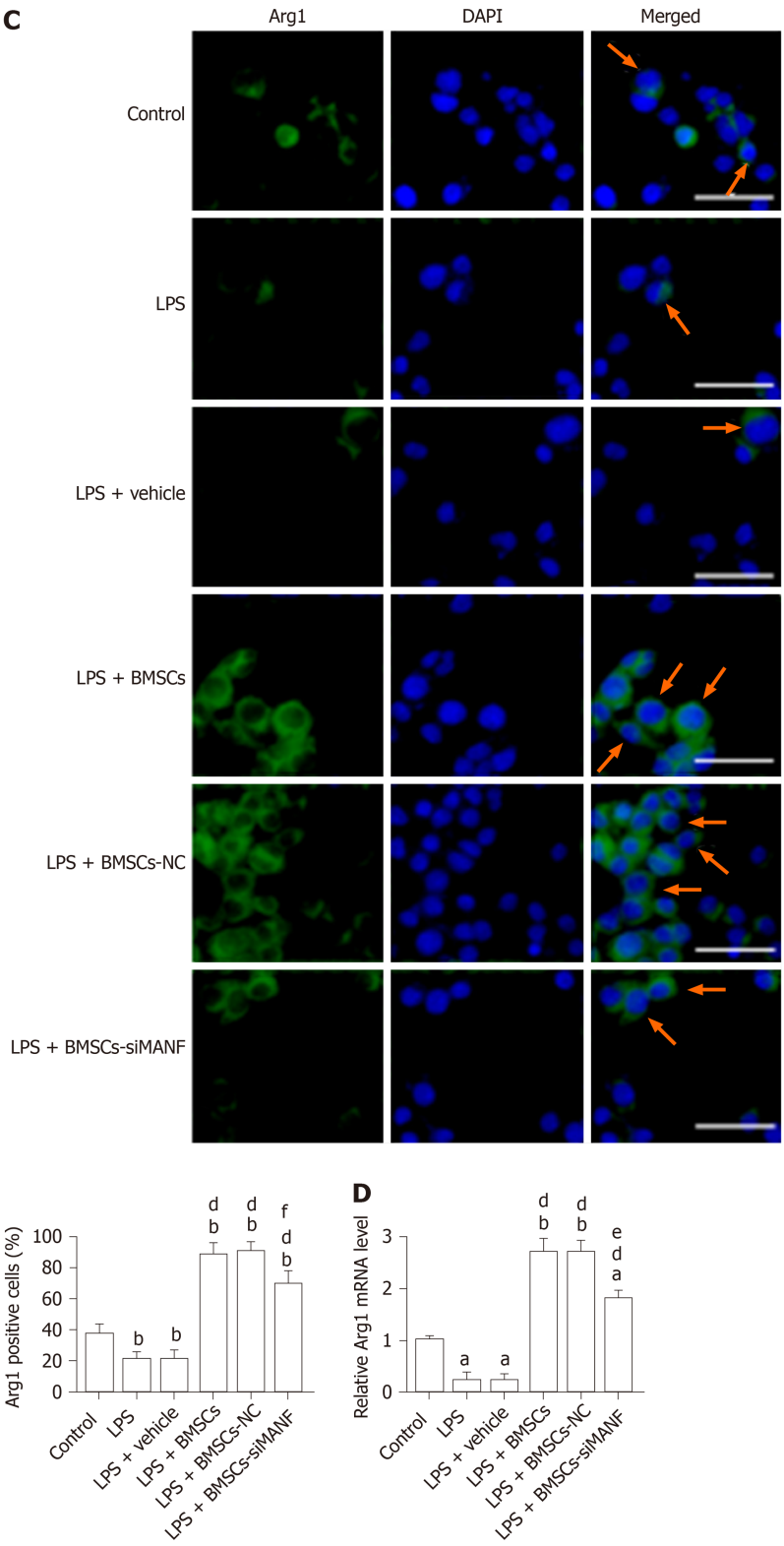


Figure 7 ***In vitro*** **analysis of M1 and M2 polarization markers following exposure to lipopolysaccharide and bone marrow mesenchymal stem cells.** A and B: Immunocytochemistry (A) and qRT-PCR analysis (B) of iNOS expression in microglia. Representative images of immunofluorescence staining for iNOS (green), with DAPI (blue) as a nuclear counterstain, are shown. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* LPS and LPS + vehicle groups; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* LPS + BMSCs and LPS+BMSCs-NC groups. Scale bar = 50 μm. Arrows point to the iNOS^{+} cells; C and D: Immunocytochemistry (C) and qRT-PCR analysis (D) of Arg1 expression in LPS-stimulated microglia in the presence or absence of BMSCs. Representative images of immunofluorescence staining for Arg1 (green), with DAPI (blue) as a nuclear counterstain, are shown. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs* Control group; $^{d}P < 0.001$ *vs* LPS and LPS + vehicle groups; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* LPS + BMSCs and LPS+BMSCs-NC groups. Scale bar = 50 μm. Arrows point to the Arg1^{+} cells. The values are expressed as the mean ± SD (n = 6). LPS: Lipopolysaccharide; BMSCs: Bone marrow mesenchymal stem cells; BMSCs-NC: Negative control-transfected BMSCs; BMSCs-siMANF: MANF siRNA-transfected BMSCs; iNOS: Inducible nitric oxide synthase; Arg-1: Arginase-1; DAPI: 4'6-diamidino-2-phenylindole.

integration and repair[42,43]. Strategy switching of the cerebral environment from a

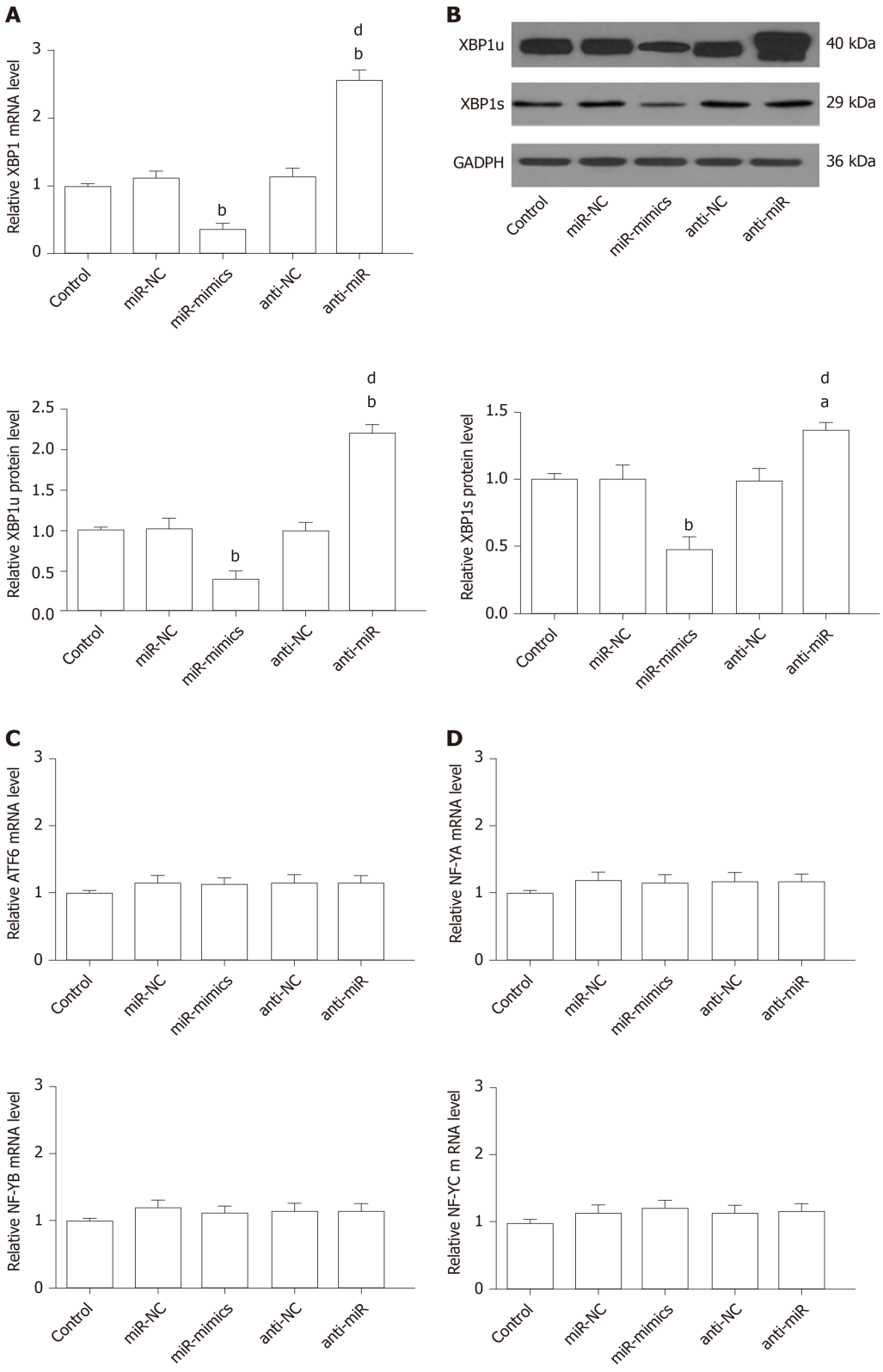
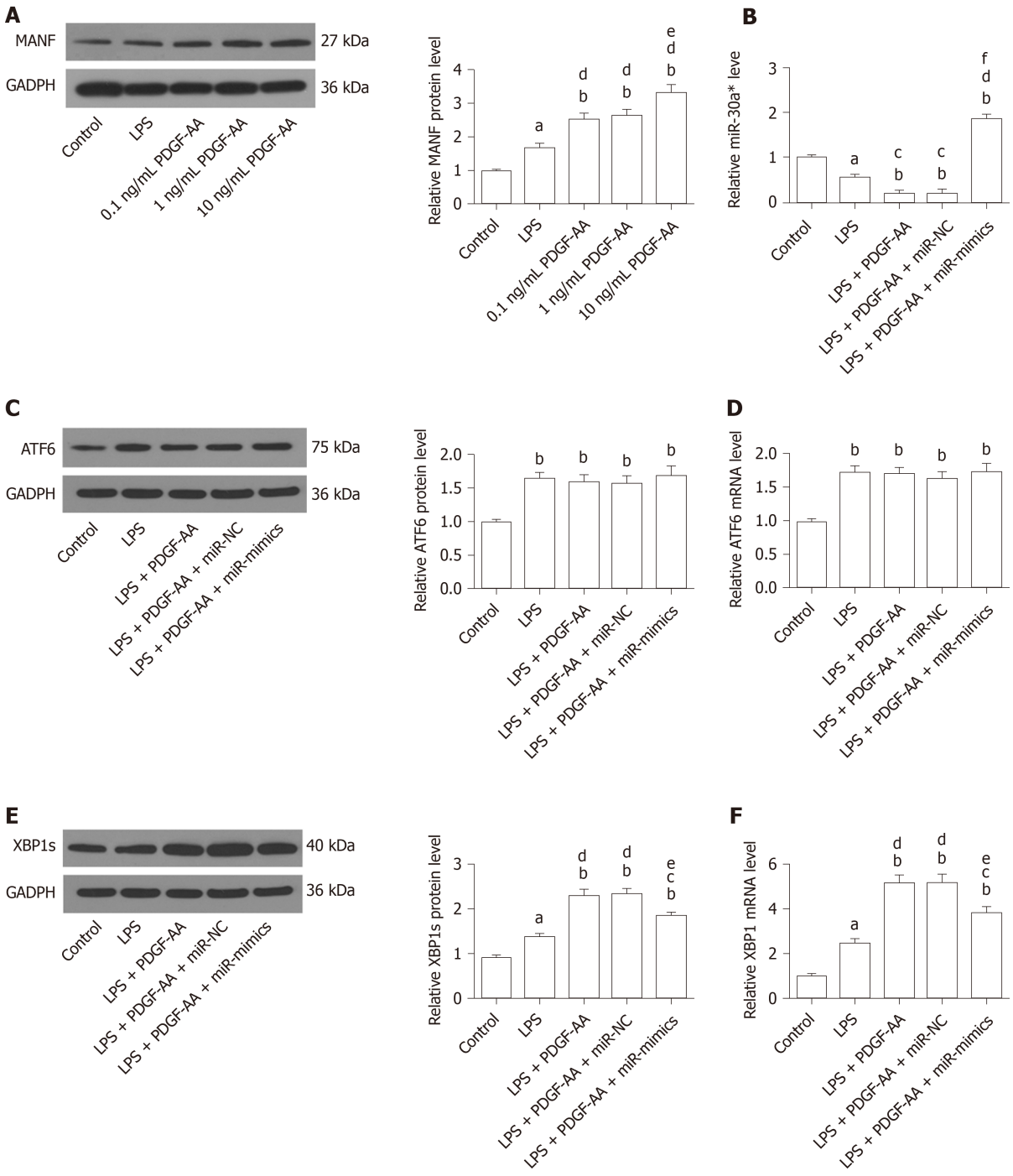


Figure 8 miR-30a* inhibits X-box binding protein 1 expression without regulating the levels of activating transcription factor 6 and NF-YA–C in HAPI cells. A and B: qRT-PCR (A) and Western blot analysis (B) of XBP1 expression in HAPI cells transfected with miR-mimics, anti-miR, or corresponding NC oligonucleotides; C and D: qRT-PCR analysis of ATF6 (C) and NF-YA–C (D) expression in HAPI cells after transfection of miR-mimics, anti-miR, or corresponding NC oligonucleotides. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control, miR-NC and anti-NC groups; $^{d}P < 0.01$ *vs* miR-mimics group. The values are expressed as the mean ± SD ($n = 6$). XBP1: X-box binding protein 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ATF6: Activating transcription factor 6; NF-Y (A,B,C): Nuclear factor-Y (A,B,C); miR-NC: miR-30a*-negative control-transfected microglia; miR-mimics: miR-30a*-mimics-transfected microglia; anti-NC: anti-miR-30a*-negative control-transfected microglia; anti-miR: miR-30a*-inhibitor-transfected microglia.

proinflammatory toxic to an anti-inflammatory and neuroprotective condition could be a potential therapy for ischemic stroke. BMSCs therapy is the desired approach for addressing this issue. Therefore, BMSCs-regulated microglia/macrophage polarization is crucial for transplantation of stem cells after cerebral ischemia.

Although MANF was initially discovered by its neurotrophic activity, further studies revealed that MANF could have an immunomodulatory effect on macrophage differentiation in the retina and spleen[29,44]. Our recent study has demonstrated that the therapeutic effects on stroke are probably related to the paracrine function of BMSCs[45]. Due to the anti-inflammatory property of MANF, a secretory neurotrophic factor, we constructed genetically modified BMSCs as vehicles that downregulated MANF expression for this cell transplantation in MCAO rats. In earlier studies, MANF was shown to be induced at the early stage of brain ischemia, decreasing to the control level at 1 wk after ischemia[17,46]. The protein levels of MANF started to increase at 3 h, and peaked at 24 h in a rat model of intracerebral or subarachnoid hemorrhage[47,48]. These results indicated that MANF was induced by ER stress in a time-dependent manner. Therefore, as the time point for exploring the role of MANF in BMSCs-mediated M2 polarization, 24 h of reperfusion after 2 h of ischemia was reasonable. M1 phenotype macrophages express high levels of iNOS that compete with Arg1 for L-arginine, the common substrate of both enzymes. Switching the L-arginine metabolism from iNOS to Arg1 is vital to limit NO production and downregulate inflammation[49]. Thus, iNOS and Arg1 represent the classical M1 and M2 phenotypes, respectively. Temporal analysis of cell phenotypes in ischemic animals has demonstrated that microglia/macrophages respond dynamically to ischemic injury, experiencing an early healthy M2 phenotype, followed by a transition to a sick M1 phenotype[5]. In keeping with time-dependent MANF expression in a rat brain ischemia model, MANF might play a critical role in the M2-to-M1 shift, highlighting the importance of BMSCs transplantation at the early stage of ischemic stroke. Previous studies have demonstrated that MANF expression is upregulated in the activated microglial cells, without investigating whether and how MANF modulates microglia polarization[50]. In this study, we found that MANF released from BMSCs acted directly on microglial cells and macrophages, which functioned both as sources and targets of MANF. Importantly, we observed that MANF secreted by BMSCs directly induced M2 microglia/macrophage polarization, which explained the mechanism of BMSCs-induced immunomodulatory effects. Although we performed the short-term behavioral tests in the MCAO rats, our data showed that BMSCs treatment promoted functional recovery and decreased the infarct volume caused by MCAO *via* production of MANF. Therefore, the cytoprotective and M2-inducible functions of MANF paracrine signaling are likely to synergize to promote tissue recovery. Meanwhile, we noted that MANF had overlap with DAPI, suggesting that MANF localizes in the nucleus and focal ischemia induced its relocalization, which indicated that MANF might be a negative regulator of inflammation and mediate the crosstalk with the nuclear factor (NF)-κB pathway[51]. In contrast, LPS-induced MANF expression was localized in the cytoplasm, whereas nuclear MANF immunopositive signals were not observed. The reason why MANF exhibits different cellular distribution under different pathological conditions is worthy of further investigation.

In this study, inhibition of MANF significantly decreased, but did not eliminate, the effect of BMSCs on microglia/macrophage polarization. To explore other soluble mediators responsible for interactions between BMSCs and microglia/macrophages, the PDGF-AA/MANF axis was investigated. We illustrated a clear dose-responsive effect of PDGF-AA on induction of MANF in microglia. The maximal induction dose of PDGF-AA was 10 ng/mL, however, low concentrations of PDGF-AA could enhance MANF expression as well. There is growing evidence supporting the concept that secretion of PDGF-AA, but not -AB and -BB ligand, by BMSCs promotes M2 polarization and neurorestoration in MCAO rats[11]. Although we were unable to determine the PDGF-AA/MANF signaling pathway *in vivo*, we speculate that the indirect (induction of MANF expression in microglia/macrophages) activity of PDGF-AA contributes to its tissue repair effect against ischemic injury. Meanwhile, we found that reduction of MANF secreted by BMSCs did not influence PDGF-AA expression, suggesting that the molecular mechanism of BMSC-mediated activation of PDGF-AA expression involves some other soluble factors, at least not the downstream signaling pathways of MANF. In addition, as a transcription factor, XBP1 activation plays a key role in the UPR, which has been proven to recognize and bind to ERSE and ERSE-II, whereas both exert different effects on MANF transcription in mice and humans[26,52]. A previous study has shown that MANF may in turn regulate the function of XBP1s at the protein level in the UPR, but does not affect the transcriptional levels of XBP1[19], which is in accordance with our study. In this investigation, PDGF-AA treatment


A
MANF
GADPH
27 kDa
36 kDa
Control
LPS
0.1 ng/mL PDGF-AA
1 ng/mL PDGF-AA
10 ng/mL PDGF-AA
Relative MANF protein level
B
Relative miR-30a* leve
LPS + PDGF-AA
LPS + PDGF-AA + miR-NC
LPS + PDGF-AA + miR-mimics
C
ATF6
GADPH
75 kDa
36 kDa
Relative ATF6 protein level
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E
XBP1s
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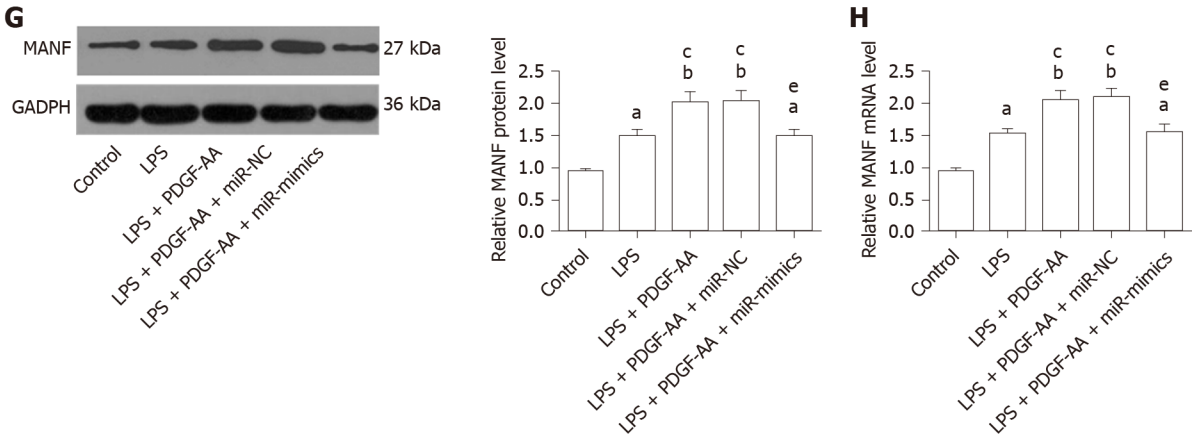

Figure 9 Platelet-derived growth factor-AA-mediated induction of mesencephalic astrocyte–derived neurotrophic factor involves miR-30a*/X-box binding protein 1 signaling in HAPI cells stimulated by lipopolysaccharide. A: Western blot analysis of PDGF-AA-induced MANF expression in activated HAPI cells. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control group; $^{d}P < 0.01$ *vs* LPS group; $^{e}P < 0.05$ *vs* 0.1 ng/mL and 1 ng/mL PDGF-AA groups; B: qRT-PCR analysis of miR-30a* expression in untreated or 10 ng/mL PDGF-AA-treated activated HAPI cells transfected with or without miR-mimics or miR-NC; C and D: Western blot (C) and qRT-PCR analysis (D) of ATF6 expression in untreated or 10 ng/mL PDGF-AA-treated activated HAPI cells transfected with or without miR-mimics or miR-NC; E and F: Western blot (E) and qRT-PCR analysis (F) of XBP1 expression in untreated or 10 ng/mL PDGF-AA-treated activated HAPI cells transfected with or without miR-mimics or miR-NC; G and H: Western blot (G) and qRT-PCR analysis (H) of MANF expression in untreated or 10 ng/mL PDGF-AA-treated activated HAPI cells transfected with or without miR-mimics or miR-NC. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* LPS group; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* LPS + PDGF-AA and LPS + PDGF-AA + miR-NC groups. The values are expressed as the mean ± SD ($n = 6$). MANF: Mesencephalic astrocyte–derived neurotrophic factor; PDGF-AA: Platelet-derived growth factor-AA; XBP1: X-box binding protein 1; ATF6: Activating transcription factor 6; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; LPS: Lipopolysaccharide; miR-NC: miR-30a*-negative control-transfected microglia; miR-mimics: miR-30a*-mimics-transfected microglia.

significantly increased expression of XBP1s protein and XBP1 mRNA, but not ATF6 in LPS-induced ER stress processes of microglia. Therefore, having a key role in promoting MANF expression, XBP1 is the downstream target of PDGF-AA.

A few ER-stress-inducible miRNAs have been identified and shown to negatively regulate translation of certain secretory pathway proteins, suggesting that miRNAs play integral roles in the UPR[53,54]. Recently, miR-30a has been reported to be an important regulator of the inflammatory response in microglia[55]. To clarify whether miR-30a* has similar effects to miR-30a, we further established the essential roles of miR-30a* in XBP1 expression in microglia. Our results revealed that miR-30a* was downregulated and this downregulation increased XBP1 expression, resulting in upregulation of MANF in the LPS-induced microglia. Importantly, we found that PDGF-AA treatment decreased miR-30a* expression, leading to the enhanced expression of XBP1 and its downstream target MANF. A recent study confirmed that NF-κB played a critical role in upregulating expression of miR-30c-2* in UPR[32]. Based on this, we cannot exclude the possibility that MANF-induced activity of PDGF-AA partially contributes to its inhibitory effect against the NF-κB/miR-30a* pathway, so how PDGF-AA influences miR-30a* expression deserves further study.

To our knowledge, this is the first study to demonstrate that BMSCs can induce M2 phenotype microglia *via* a novel secreted factor, MANF, both *in vivo* and *in vitro*. We noticed a synergistic relation between PDGF-AA and MANF secreted by BMSCs, which led to upregulation of MANF in microglia that then promoted M2 polarization. Our data provide a link between a miRNA and direct regulation of the ER stress response and reveal a novel molecular mechanism by which the PDGF-AA pathway, *via* miR-30a*, influences XBP1-mediated MANF expression in the UPR. To date, the receptor and the signaling pathway for MANF are still obscure[56], although protein kinase C signaling has been activated downstream of MANF[57]. Therefore, how MANF functions in a paracrine manner to induce M2 polarization needs further attention and investigation.

In conclusion, our present study demonstrated that the paracrine function of MANF may contribute to the mechanisms underlying BMSCs-derived M2 microglia/macrophage polarization. As a paracrine interaction between BMSCs and microglia through synergistic effect of MANF and PDGF-AA pathway governing M2 polarization, PDGF-AA/miR-30a*/XBP1/MANF signaling for MANF induction should be taken into account.

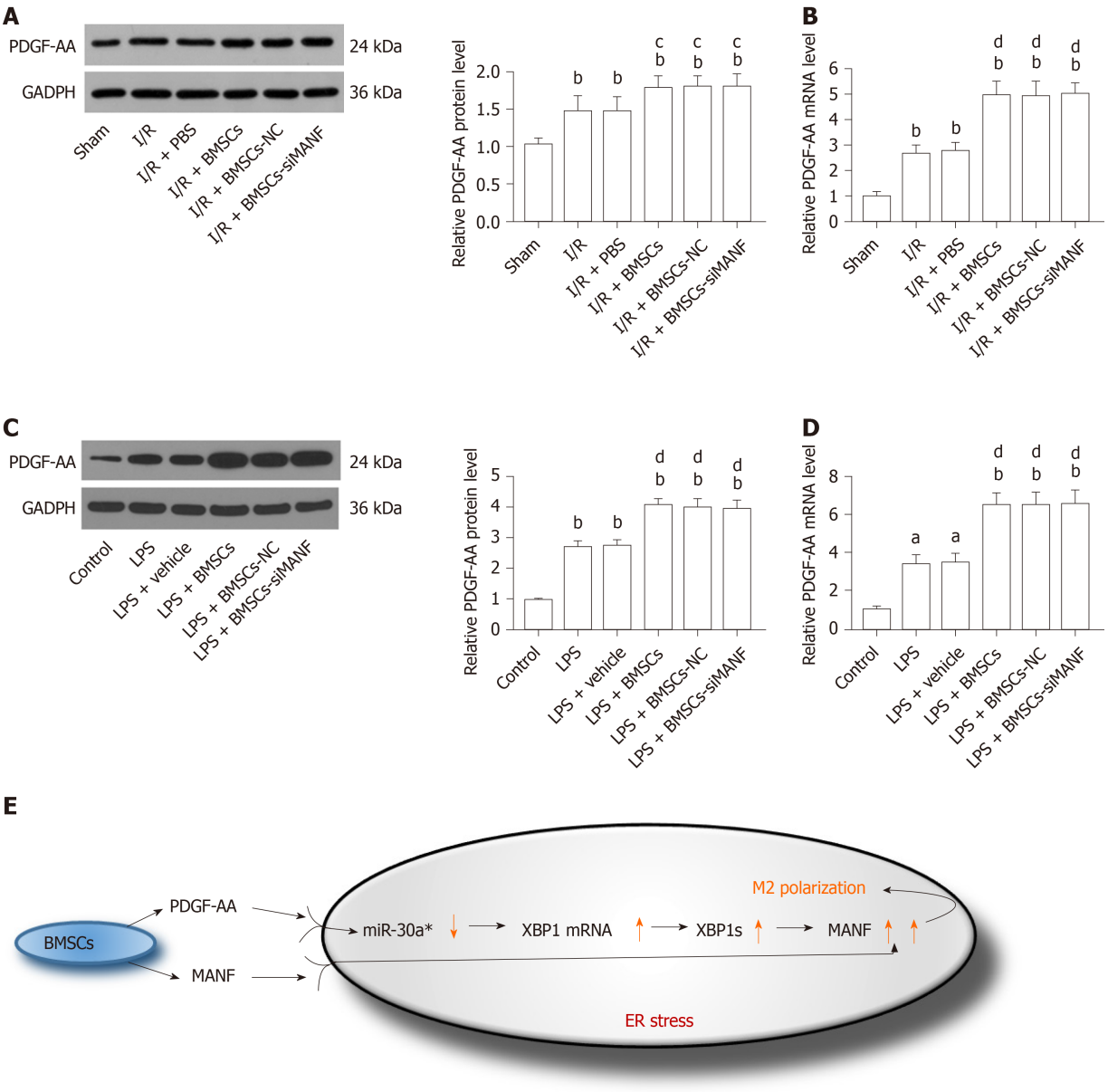

Figure 10 ***In vivo*** **and** ***in vitro*** **analysis of platelet-derived growth factor-AA expression.** A and B: Western blot (A) and qRT-PCR analysis (B) of PDGF-AA expression in the injured brains. $^{b}P < 0.01$ *vs* Sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* I/R and I/R + PBS groups; C and D: Western blot (C) and qRT-PCR analysis (D) of PDGF-AA in LPS-stimulated microglia in the presence or absence of BMSCs. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control group; $^{d}P < 0.01$ *vs* LPS and LPS + vehicle groups. The values are expressed as the mean ± SD ($n = 6$); E: Proposed mechanism for synergistic regulation of PDGF-AA/miR-30a*/XBP1/MANF pathway and MANF paracrine signaling during BMSCs-induced M2 polarization. PDGF-AA: Platelet-derived growth factor-AA; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; I/R: Ischemia/reperfusion; LPS: Lipopolysaccharide; PBS: Phosphate-buffered saline; BMSCs: Bone marrow mesenchymal stem cells; BMSCs-NC: Negative control-transfected BMSCs; BMSCs-siMANF: MANF siRNA-transfected BMSCs; XBP1: X-box binding protein 1; MANF: Mesencephalic astrocyte–derived neurotrophic factor; ER: Endoplasmic reticulum.

ARTICLE HIGHLIGHTS

Research background

Bone marrow mesenchymal stem cells (BMSCs) have been widely studied for their applications in stem-cell-based stroke therapy. Although anti-inflammatory and paracrine effects of grafted BMSCs have been shown, the precise mechanism underlying BMSCs-induced M2 microglia polarization remains unclear. Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a new member of the neurotrophic factor families, which is upregulated during endoplasmic reticulum (ER) stress and protects several cell populations from ER-stress-induced cell death *in vivo* or *in vitro*.

Research motivation

MANF and platelet-derived growth factor (PDGF)-AA/MANF signaling have been shown to have an immunoregulatory effect on M1/M2 macrophage differentiation to promote damage repair and neuroprotective effect.

Research objectives

In the present study, the aim was to detect whether MANF paracrine signaling mediated BMSCs-induced M2 polarization and to determine the molecular mechanism underlying the PDGF-AA/MANF signaling pathway.

Research methods

We first identified the secretion of MANF by BMSCs and developed genetically modified BMSCs that downregulated MANF expression. BMSCs were injected into the right striatum 24 h before cerebral ischemia/reperfusion injury. Using a rat middle cerebral artery occlusion (MCAO) model and BMSCs/microglia Transwell coculture system, the effect of BMSCs-mediated MANF paracrine signaling on M1/M2 polarization *in vivo* and *in vitro* was determined by Western blot, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and immunofluo-rescence. The transgenic microglia were used to assess the effect of miR-30a* on PDGF-AA/miR-30a*/X-box binding protein (XBP) 1/MANF signaling pathway. Western blot and qRT-PCR were conducted to examine the expression of ER stress-related markers.

Research results

In vivo or *in vitro*, BMSCs induced functional recovery and increased M2 marker expression, as well as decreased expression of M1 marker, which were inhibited by MANF siRNA treatment. As another soluble factor secreted by BMSCs, PDGF-AA upregulated XBP1 and MANF expression *via* downregulating miR-30a* in the activated microglia.

Research conclusions

BMSCs promote M2 phenotype polarization through MANF secretion, which might partially contribute to the functional outcomes in stroke rats. Besides MANF paracrine signaling, the PDGF-AA/miR-30a*/XBP1/MANF signaling pathway influences BMSCs-mediated M2 polarization.

Research perspectives

These findings will be beneficial in development of an approach with high efficiency to regulate BMSCs-induced M2 polarization, and strengthen the potential of cell therapeutics to enhance the reversal of behavioral deficits caused by ischemic stroke.

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SYSTEMATIC REVIEWS

Role of stem cell therapies in treating chronic wounds: A systematic review

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Abstract

BACKGROUND

The impairment of cutaneous wound healing results in chronic, non-healing wounds that are caused by altered wound environment oxygenation, tissue injury, and permissive microbial growth. Current modalities for the treatment of these wounds inadequately address the complex changes involved in chronic wound pathogenesis. Consequently, stem cell therapies have emerged as a potential therapeutic modality to promote cutaneous regeneration through trophic and paracrine activity.

AIM

To investigate current literature regarding use of stem cell therapies for the clinical treatment of chronic, non-healing wounds.

METHODS

PubMed, EMBASE, Cochrane Library, Web of Science, and Scopus were queried with combinations of the search terms "mesenchymal stem cells," "adult stem cells," "embryonic stem cells," "erythroid precursor cells," "stem cell therapies," and "chronic wounds" in order to find relevant articles published between the years of 2000 and 2019 to review a 20-year experience. Reference lists from the articles were reviewed to identify additional pertinent articles. Retrieved manuscripts (reviews, case reports/series, retrospective/prospective studies, and clinical trials) were evaluated by the authors for their depiction of clinical stem cell therapy use. Data were extracted from the articles using a standardized collection tool.

RESULTS

A total of 43 articles describing the use of stem cell therapies for the treatment of

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chronic wounds were included in this review. While stem cell therapies have been explored in *in vitro* and *in vivo* applications in the past, recent efforts are geared towards assessing their clinical role. A review of the literature revealed that adipose-derived stem cells, bone marrow-derived stem cells, bone marrow-derived mononuclear cells, epidermally-derived mesenchymal stem cells, fibroblast stem cells, keratinocyte stem cells, placental mesenchymal stem cells, and umbilical cord mesenchymal stem cells have all been employed in the treatment of chronic wounds of various etiologies. Most recently, embryonic stem cells have emerged as a novel stem cell therapy with the capacity for multifaceted germ cell layer differentiation. With the capacity for self-renewal and differentiation, stem cells can enrich existing cell populations in chronic wounds in order to overcome barriers impeding the progression of wound healing. Further, stem cell therapies can be utilized to augment cell engraftment, signaling and activity, and resultant patient outcomes.

CONCLUSION
Assessing observed clinical outcomes, potential for stem cell use, and relevant therapeutic challenges allows wound care stakeholders to make informed decisions regarding optimal treatment approaches for their patients' chronic wounds.

Key Words: Mesenchymal stem cells; Adult stem cells; Embryonic stem cells; Erythroid precursor cells; Stem cell therapies; Chronic wounds

Core tip: Chronic wounds impose a significant burden on patients and the healthcare system with poor outcomes noted with the use of standard wound care protocols alone. Using stem cell therapies to treat these wounds results in improved cell signaling, release of growth factors and cytokines, neo-vessel formation, and immunomodulatory properties. Accordingly, patients experience enhanced healing of their formerly recalcitrant wounds. The objective of this review is to systematically evaluate the use of a wide range of stem cell therapies for the treatment of chronic wounds in order to guide providers in selecting appropriate treatment options for improved patient wound healing.

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INTRODUCTION

Cutaneous wound healing is a complex and intricate process contingent on a series of regulated factors that work in a concerted manner to repair skin injury and restore barrier function. Superficial wound healing tends to follow a predictable course, with aberrancies generally noted only in the case of underlying disease states, such as diabetes. However, impairment of the wound healing process can result in chronic, non-healing wounds. Chronic wounds that do not heal within an expected period of 3 mo[1] not only result in pain and disfigurement, but also impose a significant burden on patients and the healthcare system, with annual cost estimates approaching $30 billion in the United States alone[2]. Chronic wounds are the consequence of local tissue hypoxia, bacterial colonization, and repetitive ischemia-reperfusion injury[3]. Non-healing wounds can originate from a variety of etiologies, including arterial disease, diabetes, vasculitis, venous valve insufficiency, prior irradiation[4], and skin malignancies[5].

Overcoming the factors that cause delayed wound healing involves an assessment of underlying pathology and consideration of advanced therapeutic agents. While a variety of wound care treatment options are available, few have demonstrated efficacy in the healing of chronic wounds and restoration of tissue to its pre-injury state. In

contrast with normally healing wounds, chronic wounds exhibit increased levels of proinflammatory cytokines, reactive oxygen species (ROS), proteases, and senescent cells[6]. Traditional chronic wound care protocols rely on the debridement of necrotic or infected tissue followed by the application of wound dressings and topical agents that serve to protect the wound from infection and accelerate healing. In the case of certain chronic wounds, such as diabetic foot ulcers, offloading with external compression is crucial in order to minimize pressure placed on the wound[7]. Additionally, diabetic foot ulcers, venous leg ulcers, and open amputation wounds can be treated with negative pressure wound therapy to promote granulation tissue formation, wound area contraction, primary healing, and improved skin graft retention upon application to the wound bed[8]. Management of the wound edge is further performed with electromagnetic therapy, laser therapy, ultrasound therapy, and systemic oxygen therapy[9,10]. Although these therapeutic options are varied and can be tailored to each patient, these techniques have limited success and do not consistently facilitate complete wound closure.

Consequently, stem cell therapies have emerged as an exciting field of research because of their potential for the treatment of non-healing wounds. Notably, these wounds can be characterized by damaged or depleted stem cell populations[6,11]. Stem cells possess the unique capacity to self-renew and differentiate into various cell types. Moreover, stem cells can upregulate the secretion of cytokines and growth factors necessary for immunomodulation and regeneration[12], which are two critical features inadequately addressed in chronic wound healing pathogenesis. Ranging from immature pluripotent cells to more differentiated, multipotent cells, stem cells comprise a budding area of interest for improved healing of chronic wounds without the associated risks of major surgical procedures and added donor-site morbidity. The aim of the following review is to systematically review current literature that addresses the role of stem cell therapies in treating varied chronic, non-healing wounds in order to provide plastic surgeons, clinicians, and other chronic wound care stakeholders a comprehensive guide from which to select optimal therapies for improved patient outcomes.

MATERIALS AND METHODS

Literature search

A systematic literature search was conducted according to the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) guidelines[13]. Figure 1 depicts the algorithm for article identification, screening, and review. Inclusion and exclusion criteria are presented in Table 1. PubMed, EMBASE, Cochrane Library, Web of Science, and Scopus were queried to identify relevant publications, articles, and abstracts that reported stem cell applications from the years 2000 to 2019. The queries employed a combination of search terms, including "mesenchymal stem cells," "adult stem cells," "embryonic stem cells," "erythroid precursor cells," "stem cell therapies," and "chronic wounds." The search parameters are described in detail in Supplementary Table 1. To eliminate bias, four authors independently screened all articles for inclusion or exclusion, and in the case of a conflict, a fifth author screened pertinent articles as a tiebreaker. Retrieved publications included systematic reviews, literature reviews, case reports and series, retrospective and prospective studies, and clinical trials. All studies that contained material applicable to the clinical use of stem cell therapies were reviewed, and data were extracted using a standardized collection tool. A total of 43 articles describing the use of stem cell therapies for the treatment of chronic wounds were included in this review[14-56].

RESULTS

While stem cell therapies have been extensively explored in *in vitro* and *in vivo* settings, more recent investigative efforts have attempted to assess their clinical translatability. Transplanted cells deliver cytokines, chemokines, and growth factors, induce angiogenesis and innervation, and alter the wound inflammatory process[57]. To better assess the advantages of these stem cell properties in treating patients' chronic wounds, we extracted data from studies that explore stem cell therapies in a clinical setting. Table 2 presents these treatment options, characteristic surface markers, indications for use, and mechanisms of action. Table 3 delineates the clinical outcomes

Table 1 Inclusion and exclusion criteria

Inclusion criteria
Articles published in English
Stem cell therapies with demonstrated utility in the healing of chronic wounds either alone or as a complementary modality
Systematic reviews, literature reviews, case reports, case series, retrospective and prospective studies, and clinical trials between the years 2000-2019
Exclusion criteria
Articles not published in English
No full text availability
No report of stem cell therapy application
Animal or non-human, *ex vivo*, or *in vitro* studies
Letters, comments, and editorials

observed, considerations for stem cell therapy optimization, and pertinent challenges associated with each therapy's usage.

Adipose-derived stem cells

Adipose-derived stem cells (ADSCs) are mesenchymal stem cells that comprise a pluripotent, heterogenous cell population within human adipose tissue. The popularity of ADSCs can be attributed to their ease of harvest and limited donor-site morbidity; they can be isolated *via* liposuction aspiration or excision of fat samples[27]. The most common approach for isolating ADSCs involves collagenase digestion followed by centrifugal density gradient separation[58]. Isolated ADSCs can then be expanded in monolayer cultures on standard tissue dishes with a basal medium of 10% fetal bovine serum[59]. Clinical use of ADSCs requires *in vitro* expansion that complies with good manufacturing practice (GMP) guidelines to ensure that no xenogeneic components are cultivated. As ADSCs are mesenchymal stem cells, their surface markers include CD90, CD105, CD73, CD44, and CD166 without the expression of hematopoietic cell surface markers CD34 and CD45[60].

ADSCs have demonstrated clinical efficacy in the treatment of chronic wounds secondary to severe radiation injury, chronic fistulae, and ulceration, which includes venous leg ulcers. Mechanistically, this stem cell population facilitates angiogenesis, augments the secretion of growth factors and cytokines, and allows for human dermal fibroblast proliferation through direct cell contact and paracrine activation during the re-epithelialization phase of wound healing[61]. Notably, when combined with skin substitute containing human extracellular matrix (ECM), ADSCs permit regeneration of subcutaneous, dermal, and epidermal tissues[62].

ADSCs are among the more robustly explored stem cell therapy type in the clinical treatment of chronic wounds. They have demonstrated results of improved wound healing and closure, tissue ultrastructure and hydration, neo-vessel formation, and patient symptomatology of pain and claudication[14,63-70]. In the treatment of chronic ulcers secondary to peripheral arterial disease, Marino *et al*[71] observed decreased ulcer size, depth, pain, and improved transcutaneous saturation in all patients over the course of treatment. The versatility of ADSCs is further highlighted in treating chronic Crohn's fistulas; treated patients experienced improved wound healing seen over 8 wk without the incidence of adverse events[72]. Akita *et al*[14] found that the use of an artificial dermis (Terudermis, Japan) scaffold protected ADSCs from infection and ambient dryness, while Larsen *et al*[43] found improved ulcer re-epithelialization and healing when ADSCs were administered on an OASIS wound matrix in conjunction with compression therapy. In addition, seeding ADSCs in hydrogel delivery vehicles by capillary force and then administering these hydrogel systems *in vivo* effectively enhances stem cell genetic expression and survival for improved wound healing outcomes[73]. When harvesting ADSCs from subcutaneous adipose tissue, it is important to avoid penetration of the deeper visceral cavity or underlying major muscles, vessels, and nerves. Upon injection of ADSCs to chronically radiated wound beds, care must also be taken to avoid surface rupture or laceration[14].

Though their clinical potential has been favorably evidenced, there are some challenges to consider with the use of ADSCs. This cell population is not immortal and displays signs of aging and loss of chemokine markers with repeat culturing[50]. Further, adipose tissue can widely vary in its metabolic activity and capacity for

Table 2 Stem cell therapies

Stem cell therapy	Cell markers	Indications	Mechanisms of action
Adipose-derived stem cells (ADSCs)	CD90+, CD105+, CD73+, CD44+, CD166+; CD34-, CD45-	Severe radiation injury, chronic ulcers, venous leg ulcers, chronic fistulae	Kim *et al*[61]: Promote angiogenesis, secrete growth factors and cytokines, and allow for human dermal fibroblast proliferation through direct cell contact and paracrine activation in the re-epithelialization phase; Trottier *et al*[62]: When combined with skin substitute with human extracellular matrix (ECM), ADSCs produce subcutaneous, dermal, and epidermal regenerated tissues
Bone marrow-derived stem cells (BMMSCs)	CD105+, CD73+, CD90+; CD13-, CD34-, CD45-	Severe radiation-associated wounds, chronic diabetic ulcers, advanced pressure ulcers in patients with spinal cord injury, and other intractable wounds	Han *et al*[64]: Synthesize high amounts of collagen, fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF); Ren *et al*[76]: Induce proliferation and potent differentiation of cells under low oxygen tension with morphologic and cell cycle changes towards bone and fat; Stoff *et al*[77]: Increase tensile strength of postoperative incisional wounds; Maxson *et al*[46]: Secrete antimicrobial factors and promote host immune response
Bone marrow-derived mononuclear cells (BMMNCs)	CD133+, CD117+, CD34+	Chronic ulcers	Amato *et al*[15]: Secrete angiogenic growth factors to decrease local inflammation and promote vascularization
Epidermally-derived mesenchymal stem cells (EMSCs)	CD90+, CD73+, CD105+/-; CD34-, CD271-	Chronic ulcers, burns, generalized junctional epidermolysis bullosa (JEMB)	Yang *et al*[89]: Promote re-epithelialization in wound healing and regenerate functional epidermal layer
Fibroblast stem cells (FSCs)	CD34+, CD11b+, CD13+, MHC II+, CD86+, CD45+, collagen-1+, procollagen-1+; CD44-	Chronic ulcers	Amato *et al*[15]: Increase cell proliferation, ECM deposition, wound contraction, and vascularization with additional secretion of growth factors and cytokines
Keratinocyte stem cells (KSCs)	K5, K14, K15, integrins; CD34-	Chronic ulcers	Lampert *et al*[94]: Enable the formation of the stratified keratinizing epidermis; Domaszewska-Szostek *et al*[26]: Proliferate, migrate, and differentiate during re-epithelialization with mechanical, antibacterial, and nutritious roles. Keratinocytes also interact with fibroblasts during the wound healing process and tissue regeneration. They perform autocrine secretion of IL-6 and nitric oxide, release growth factors, and help restore the barrier function of skin
Placental mesenchymal stem cells (PMSCs)	CD105+, CD73+, CD90+, CD44+; CD34-, CD45-	Chronic venous ulcers	Farivar *et al*[30]: Stimulate tissue regeneration and repair for improved wound healing
Umbilical cord mesenchymal stem cells (UMSCs)	CD105+, CD73+, CD90+; CD34-, CD45-	Chronic diabetic ulcers	Hashemi *et al*[37]: Secrete growth factors for wound healing and can differentiate into fibroblast, epithelial, and endothelial cells for wound healing
Embryonic stem cells (ESCs)	Oct-4, Stage specific embryonic antigens (SSEAs)	Intractable wounds	Guenou *et al*[102]: Capable of differentiating into all three germ cell layers and can form functional human basal keratinocytes

proliferation and differentiation depending on the site of tissue harvest and patient characteristics, such as age and sex[31]. Marfia *et al*[45] identified that autologous ADSCs have an altered genotype in diabetic patients, which is characterized by decreased potency and expression of vascular endothelial growth factor-A (VEGF-A) and chemokine receptor CXCR4. Judicious use of ADSC therapy requires close patient surveillance for tumor formation as well as consideration of strategies to improve cell homing and transplantation[50].

Bone marrow-derived stem cells

Bone marrow-derived stem cells (BMMSCs) were originally isolated by Friedenstein *et al*[74] in 1966 and presently constitute a mesenchymal stem cell population that is

Table 3 Stem cell therapy clinical applications

Stem cell therapy	Clinical outcomes	Considerations for therapy optimization	Challenges associated with use
Adipose-derived stem cells (ADSCs)	Studies that demonstrated chronic wound healing: Akita *et al*[14]: No recurrences or wound abnormalities at 8 ± 2.2 wk (n = 5 patients); Lee *et al*: Wound healing rate of 66.7%, with improvement in pain and claudication walking distance (n = 15 patients); García-Olmo *et al*[72]: Epithelial covering of chronic Crohn's fistulas and healing by 8 wk (n = 4 patients); Rigotti *et al*[63]: Improved tissue ultrastructure, hydration, and neo-vessel formation in chronically radiated wounds over 31 mo (n = 20 patients); Studies that demonstrated a decrease in peripheral arterial disease ulcer size, number, and pain; Marino *et al*[71]: 6 patients had complete healing over 3 mo (n = 10 patients); Bura *et al*[106]: Improved transcutaneous saturation over 6 mo (n = 7 patients). Studies that showed higher chronic wound closure: Raposio *et al*[66]: Higher chronic wound closure than with control treatment (n = 16 patients); Carstens *et al*[67]: Complete chronic wound closure in 9 mo (n = 4 patients) with reduced pain; Han *et al*[64]: 100% chronic diabetic wound closure at 8 wk (n = 28 patients) *vs* 62% in the control group (n = 26 patients). Studies that demonstrated ulcer closure: Chopinaud *et al*[68]: Hypertensive leg ulcer closure of 93.1% at 6 mo with reduced fibrin, necrosis, and pain (n = 10 patients); Konstantinow *et al*[69]: 100% wound venous and arterial-venous ulcer closure over 6 mo (n = 13 patients) with reduced pain within days; Darinskas *et al*[70]: Complete ulcer healing (n = 15 patients) with less pain and walking improvement.	Stem cell delivery: Akita *et al*[14]: A 2-layered (atelocollagen + collagen) artificial dermis scaffold with injected ADSCs protects cells from infection and ambient dryness; Garg *et al*[73]: Capillary force ADSC seeding of hydrogels increases cell genetic expression and survival; Larsen *et al*[43]: ADSCs can be administered on an OASIS wound matrix for improved ulcer healing. Stem cell harvest: Akita *et al*[14] : ADSCs need to be cultured in very lean patients. When harvesting from subcutaneous adipose tissue, take care not to penetrate deeper viscera and vasculature. When injecting in chronic radiation injury sites, avoid surface rupture or laceration.	Gauglitz *et al*[31]: ADSCs are not immortal and display signs of "old age" when subject to culturing. Adipose tissue varies in metabolic activity and capacity for proliferation and differentiation, depending on the location of tissue harvest and other patient variables (age, gender); Marfia *et al*[45]: Autologous ADSCs have an altered genotype in diabetic patients, resulting in decreased potency, and decreased expression of vascular endothelial growth factor A and chemokine receptor CXCR4; Rezaie *et al*[50]: Short survival, poor transplantation, inferior homing, possibility of tumor formation, and loss of chemokine markers during ex vivo expansion.
Bone marrow-derived stem cells (BMMSCs)	Badiavas *et al*[78]: Demonstrated complete closure, dermal rebuilding, reduced scarring, and successful engraftment of cells over 1 year (n = 3 patients); Vojtassák *et al*[82]: Increase in dermal vascularity and dermal thickness of the wound bed after 29 d of treatment (n = 1 patient). Studies that demonstrated improved ulcer healing: Lu *et al*[79]: Improved healing in chronic diabetic ulcers at 6 wk with 100% healing 4 wk earlier than treatment with BMMNCs (n = 18 patients); Dash *et al*[80]: Improved Buerger disease (n = 9 patients) and diabetic (n = 3 patients) ulcer healing at 12 wk; Sarasúa *et al*[33]: Full healing of longstanding stage IV pressure ulcers in patients with spinal cord injury (n = 19 patients). Studies that showed decrease in chronic wound size: Gupta *et al*[35]: 70% reduction in chronic wound size over 3 wk followed by complete closure at 1 mo with application of cultured BMMSCs, rather than application of bone marrow aspirate alone (n = 19 patients); Humpert *et al*[75]: Reduction in chronic venous and neuroischemic wound size, increased vascularization, and infiltration of mononuclear cells after 7 d of treatment (n = 1 patient); Rogers *et al*[81]: Reduction in size in chronic wounds of different etiologies (n = 3 patients); Wettstein *et al*[54]: Reduction in chronic wound size of 50% over 3 wk of therapy (n = 3 patients).	Stem cell delivery: Yoshikawa *et al*[83]: Improved skin generation when BMMSCs are cultured in an artificial collagen dermis (n = 18 patients); Falanga *et al*[29]: 40% reduction in chronic wound size when BMMSCs are cultured in fibrin spray (n = 5 patients); Ravari *et al*[84]: Demonstrated significant reduction in chronic diabetic wounds after 4 wk of treatment when BMMSCs are co-administered with platelets, fibrin glue, and bone marrow-impregnated collagen matrix (n = 8 patients).	Rezaie *et al*[50]: Short survival, poor transplantation, inferior homing, possibility of tumor formation, and loss of chemokine markers during ex vivo expansion.
Bone marrow-derived mononuclear cells (BMMNCs)	Studies that demonstrated a higher wound healing rate: Yamaguchi *et al*[87]: Epidermal grafting significantly accelerated chronic diabetic foot ulcer healing (n = 10 patients); Jain *et al*[86]: Decreased wound area by 17.4% (n = 25 patients) compared to 4.84% with control (n = 23 patients) at 2 wk with average decrease of 36.4% *vs* 27.24% in wound area at 12 wk; Deng *et al*[24]: Demonstrated wound healing rate of 34.55% ± 11.18%, compared to control wound healing of 10.16% ± 2.67% (n = 10 patients); Deng *et al*[25]: Demonstrated wound healing of 26.5% ± 9.51% when administered as high density nanofat combined with negative pressure wound therapy (NPWT), compared to control healing of 12.02+4.2% with NPWT alone (n = 8 patients).	Not reported in reviewed articles.	Not reported in reviewed articles.
Epidermally-derived mesenchymal stem cells (EMSCs)	Bauer *et al*[90]: Regeneration of functional epidermis with gene-corrected EMSCs in previously infected, non-healing chronic ulcers due to junctional epidermolysis bullosa (n = 1 patient).	Teng *et al*[52]: Cultured epidermal autografts enriched with EMSCs on an ECM-compatible substrate can overcome EMSC deficiency in chronic wounds and provide ECM materials to stabilize the wound site.	Not reported in reviewed articles.
Fibroblast stem cells (FSCs)	Yamada *et al*[107]: Chronic wound size reduction of 33.3% (n = 5 patients); You *et al*[105]: Complete diabetic foot ulcer healing in 84% of patients (n = 26 patients) over 36.4+17.6 d.	Stem cell delivery: Brower *et al*[20]: Enhanced cell adherence, proliferation, and migration when delivered in a 2-chamber fibrin sealant; Yonezawa	Not reported in reviewed articles.

		et al[92]: Improved wound healing of 92% (*n* = 13 patients) when fibroblasts were cultured on a hyaluronic acid and atelo-collagen matrix; Marcelo *et al*[108]: More complete wound healing achieved when administered with autologous fibrin glue over the ulcer site.	
Keratinocyte stem cells (KSCs)	Studies that demonstrated improved wound healing: Vanscheidt *et al*[95]: Complete and faster wound healing in 38.3% of patients receiving autologous keratinocytes, compared to 22.4% in the control group (*n* = 44 patients); Moustafa *et al*[96]: improved wound healing over 6 wk when administered as cultured autologous keratinocytes on cell-free discs (*n* = 12 patients); Bayram *et al*[99]: Reduction in diabetic foot wound size of 92% (*n* = 20 patients), compared to control wound size reduction of 30%. Studies that demonstrated complete ulcer healing: Hartmann *et al*[97]: Complete ulcer healing at a mean of 14.5 wk when administered as keratinocytes transplanted in a fibrin carrier (*n* = 4 patients); De Luca *et al*[98]: Complete ulcer healing in 20 wk (*n* = 20 patients) with a 30%-84.4% reduction in size after 3 wk (*n* = 4 patients); Beele *et al*[100]: Demonstrated complete venous leg ulcer closure within 1 wk (*n* = 11 patients) over a period of 4.1-24.9 wk with decreased pain.	Stem cell delivery: Hartmann *et al*[97]: Better keratinocyte graft fixation and epithelial monolayer formation with low-density fibrin; Bayram *et al*[99]: More effective delivery when cultured keratinocytes are attached to microcarriers made of polyethylene and silica; Teepe *et al*[109]: Radial progression toward wound closure when administered as cryopreserved cultured allografts (*n* = 43 patients); Shukla *et al*[101]: Complete chronic wound healing over 12-48 wk when administered as keratinocytes along with epidermal cell suspension (*n* = 12 patients).	Rezaie *et al*[50]: Difficult to isolate, short lifespan during serial cultivation, and possible tumor formation.
Placental mesenchymal stem cells (PMSCs)	Farivar *et al*[30]: Complete chronic venous ulcer healing in 53% (*n* = 21 patients with 30 total ulcers) with 79% mean reduction in wound surface area over an average of 10.9 wk.	Farivar *et al*[30]: Improved cell survival when MSCs are administered in cryopreserved, aseptic placental tissue (hVWM).	Duscher *et al*[27]: Appropriate donor selection is necessary to avoid immune-mediated rejection or transmission of genetic diseases. More efficient cell isolation, culture, and expansion techniques are needed, along with close surveillance for malignant transformation.
Umbilical cord mesenchymal stem cells (UMSCs)	Hashemi *et al*[37]: Significant decrease in chronic diabetic ulcer size over 9 d with decreased wound healing time (*n* = 5 patients).	Hashemi *et al*[37]: Improved tissue regeneration and wound healing when cells are seeded on an acellular amniotic membrane scaffold.	Duscher *et al*[27]: Appropriate donor selection is necessary to avoid immune-mediated rejection or transmission of genetic diseases. More efficient cell isolation, culture, and expansion techniques are needed, along with close surveillance for malignant transformation.
Embryonic stem cells (ESCs)	Not reported in reviewed articles.	Not reported in reviewed articles.	Okano *et al*[104]: Patients will need to be monitored for tumorigenesis and teratoma formation.

typically harvested *via* iliac crest aspiration. This aspirate is then subjected to *in vitro* selection, cell expansion in culture, and topical application to wounds for tissue regeneration. BMMSCs display the typical mesenchymal stem cell surface markers, including CD105, CD73, and CD90. Their clinical utility encompasses the treatment of severe radiation-associated wounds, chronic diabetic ulcers, advanced pressure ulcers, as seen in patients who have undergone spinal cord injury, and other forms of intractable wounds.

Consistent with other mesenchymal stem cell types, BMMSCs have immunomodulatory properties[46] and promote angiogenesis[75]. These stem cells synthesize high amounts of collagen, fibroblast growth factor (FGF), and VEGF[64], allow for cell proliferation and differentiation under low oxygen tension conditions[76], and demonstrate increased tensile strength when applied to postoperative incisional wounds[77]. When clinically administered, BMMSCs have shown complete closure,

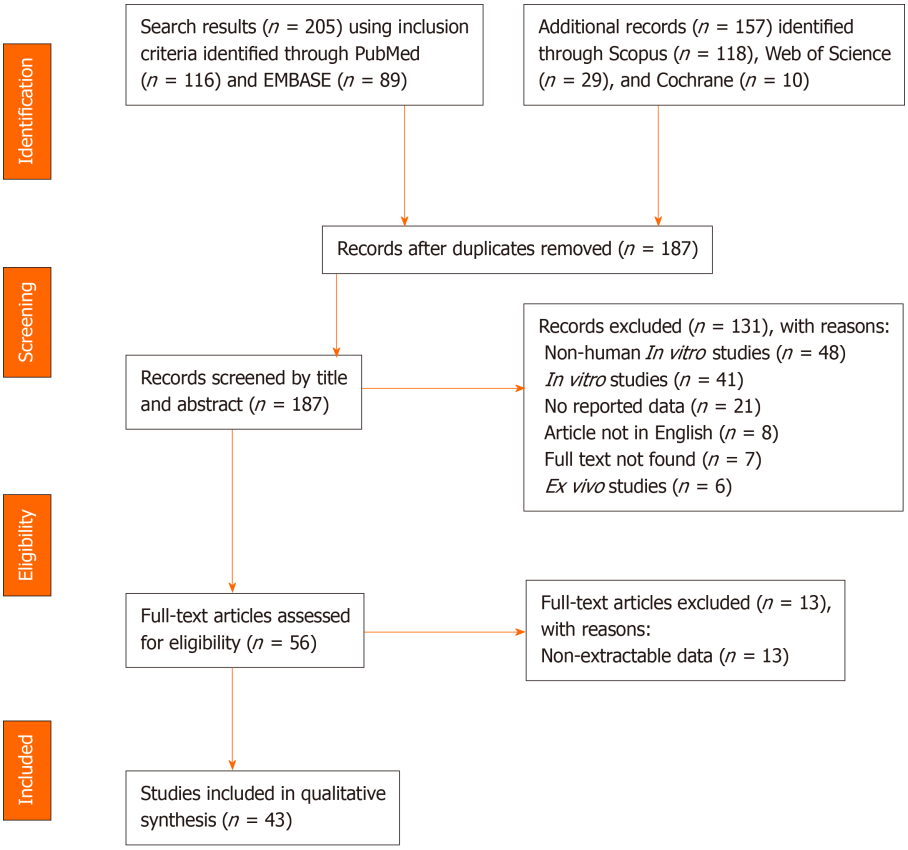

Figure 1 PRISMA Flowchart.

dermal rebuilding, reduced scarring, and successful cell engraftment in non-healing wounds[78]. Particularly in the case of chronic ulcers and wounds resultant from traumatic, thermal, electric, or infectious etiologies, this stem cell therapy is shown to significantly reduce wound size[29,35,54,79-82]. For chronic diabetic ulcers, BMMSC therapy results in improved limb perfusion, ankle-brachial indices, transcutaneous oxygen pressure, and magnetic resonance angiography analysis[79]. Sarasúa *et al*[33] found that BMMSC treatment of longstanding stage IV pressure ulcers in patients with spinal cord injury decreased the mean hospital stay from 85.16 to 43.06 d, when compared with standard treatment modalities alone. In addition, patients given the BMMSC treatment did not evidence any ulcer recurrence over a follow-up period of 19 mo.

In order to improve the delivery and outcomes associated with BMMSC therapy, these cells can be cultured and placed in an artificial collagen dermis as a composite graft, which can improve skin regeneration processes[83]. Additionally, when BMMSCs are cultured and administered *via* a fibrin spray, chronic wound or ulcer size is reduced significantly by 40% over a period of 20 wk, with no adverse events reported[29]. To enhance their favorable therapeutic profile, BMMSCs can be administered along with platelets, fibrin glue, and bone marrow-impregnated collagen matrix; this strategy has resulted in significant diabetic wound closure in patients with formerly recalcitrant wounds[84]. Similar to ADSCs, BMMSCs necessitate careful monitoring of patients for possible tumor formation as well as appropriate but not excessive culturing in order to avoid transplantation of aged cells with inferior homing abilities and loss of chemokine markers[50].

Bone marrow-derived mononuclear cells

Bone marrow-derived mononuclear cells (BMMNCs) are a heterogeneous group of cells that include mature B cells, T cells, monocytes, and a smaller proportion of progenitor cells, including hematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, and embryonic-like cells[85]. BMMNCs offer ease of harvest, processing, and administration, making them a favorable option for clinical testing. Cell surface markers include CD133, CD117, and CD34, and BMMNCs have been clinically applied most frequently in the treatment of chronic ulcers. Their most notable property is the ability to secrete angiogenic growth factors that decrease local inflammation and promote vascularization[15].

BMMNCs simultaneously accelerate the rate of wound healing and decrease wound area compared to non-stem cell therapy treatment[24,86]. When applied with epidermal grafting for the treatment of chronic diabetic foot ulcers, Yamaguchi *et al*[87] observed significant ulcer healing without incidence of osteomyelitis or the need for patient amputation. Moreover, when administered in conjunction with high density nanofat and negative pressure wound therapy, Deng *et al*[25] reported improved patient wound healing outcomes as well as decreased lymphocyte recruitment, higher collagen deposition, and increased vessel growth.

Epidermally-derived mesenchymal stem cells

Epidermally-derived mesenchymal stem cells (EMSCs) categorize the mesenchymal stem cell population present within the epidermis that is responsible for homeostasis of the superficial skin layers[88]. This population includes interfollicular, sebaceous gland, and bulge area stem cells. EMSCs are characterized by the cell surface markers CD90 and CD73, with variable expression of CD105. Clinically, this stem cell therapy has been employed to treat chronic ulcers, burns, and non-healing wounds secondary to junctional epidermolysis bullosa (JEMB). EMSCs promote both re-epithelialization in wound healing and regeneration of a functional epidermal skin layer[89]. Of note, if EMSCs undergo gene correction, they promote enhanced functional epidermal regeneration in JEMB ulcers[90]. Teng *et al*[52] found that cultured epidermal autografts enriched with EMSCs on an ECM-compatible substrate not only replenished EMSCs in chronic wounds, but also stabilized the ECM substrate within the wound site. As a stem cell category, EMSCs can be manipulated and stimulated *via* biomaterial-based approaches to modify their spatial and temporal cues for precise niche conditions that are beneficial in wound healing and skin regeneration[27].

Fibroblast stem cells

Fibroblast stem cells (FSCs) are part of a relatively novel focus in regenerative medicine and can be generated by reprogramming adult fibroblasts into an immature, pluripotent state[91]. These autologous induced pluripotent stem cells (iPSCs) are furthermore nonimmunogenic. The addition of fibroblasts to the chronic wound healing environment addresses the deficiency of appropriately functioning fibroblasts in the setting of this chronic inflammatory state. In non-healing wounds, fibroblasts exhibit premature, stress-induced cellular senescence with decreased proliferative potential, impaired reactivity to growth factors, and abnormal protein production[26]. Fibroblasts and their stem cell equivalent states have been employed in the treatment of chronic ulcers, and their characteristic cell surface markers include CD34, CD11b, CD13, MHC II, CD86, CD45, collagen-1, and procollagen-1. This cell population functions to promote proliferation, ECM deposition, wound contraction, vascularization, and secretion of growth factors and cytokines[15].

Clinically, FSCs have been shown to be more effective when co-delivered or co-cultured with additives, such as fibrin glue[57]. Brower *et al*[20] found that commercially available 2-chamber fibrin sealants, containing fibrinogen and thrombin, enhanced the adherence, proliferation, and migration of fibrocytes. Furthermore, fibroblasts have also demonstrated improved wound healing when cultured on hyaluronic acid and atelo-collagen matrices[92] prior to application to chronic wound sites.

Keratinocyte stem cells

Keratinocytes comprise the majority of cells within the human epidermis and promote re-epithelialization *via* proliferation, migration, and differentiation[93]. Keratinocyte stem cells (KSCs) reside in the basal epidermis, hair follicles, and sebaceous glands and can serve to replenish depleted keratinocyte populations found in chronic wounds. Characteristic cell surface markers of KSCs include K5, K14, K15, and integrins, and KSCs have been most widely applied in the treatment of chronic ulcers. These stem cells enable the formation of a stratified, keratinizing epidermis[94], contribute to the process of re-epithelialization, and offer antibacterial and nutritious roles[26]. When differentiated into keratinocytes, these cells interact with fibroblasts and perform autocrine secretion of interleukin-6 (IL-6) and nitric oxide, release growth factors, and facilitate the restoration of the skin's barrier function.

When administered to patients with chronic wounds, keratinocytes promote complete wound healing over a shorter period of time[95-99] compared to standard wound healing therapy, with additionally decreased local wound pain[100]. Moreover, keratinocytes can be cultured on cell-free discs[96], administered as cryopreserved cultured allografts, or suspended with epidermal cells in order to improve chronic wound healing outcomes[101]. Graft fixation can be performed with low-density fibrin

to ensure better cell survival and epithelial monolayer formation, thereby optimizing keratinocyte therapy[97]. Bayram *et al*[99] found that cultured keratinocytes attached to microcarriers of polyethylene and silica resulted in better cell delivery than keratinocytes administered alone. When considering KSC or keratinocyte therapy, it is important to recognize that these cells can be difficult to isolate, display a short lifespan throughout serial cultivation, and necessitate patient monitoring for tumor formation[50].

Placental mesenchymal stem cells

Placental mesenchymal stem cells (PMSCs) are another member of the mesenchymal stem cell category with clinical benefits. PMSCs stimulate the wound healing process through the release of trophic mediators, promotion of new vessel formation, recruitment of endogenous progenitor cells, and facilitation of cell differentiation, proliferation, and ECM formation[27]. As with other mesenchymal stem cells, PMSC surface markers include CD105, CD73, CD90, and CD44. PMSCs have been clinically investigated in the treatment of chronic venous ulcers with resultant higher quality tissue regeneration and repair[30].

When administered as cryopreserved placental tissue containing PMSCs, Farivar *et al*[30] demonstrated improved chronic venous ulcer healing, compared to standard therapy, with a significant reduction in size from baseline. PMSC therapy can be optimized when the cells are cryopreserved in aseptic placental tissue to protect placental tissue components, including growth factors and collagen-rich extracellular membranes, from degradation[30]. These stem cells require careful donor selection to avoid immune rejection or genetic disease transmission. Additionally, patient surveillance is imperative to monitor for malignant transformation[27].

Umbilical cord mesenchymal stem cells

Umbilical cord mesenchymal stem cells (UMSCs) represent another mesenchymal stem cell population aptly isolated from umbilical cord-lining tissue. Notably, umbilical cord epithelial cells have stem-cell like properties and can form stratified epithelium[27]. As with placental-derived mesenchymal stem cells, UMSCs are extracted from an extra-fetal source, and donors must be appropriately selected to avoid immune-mediated rejection or transmission of genetic diseases. Characteristic cell surface markers include CD105, CD73, and CD90. UMSCs secrete growth factors for wound healing and are capable of differentiating into fibroblast, epithelial, and endothelial cell subtypes for improved wound healing[37].

UMSCs have been employed in the treatment of chronic diabetic ulcers and significantly decrease both ulcer size and time required for wound healing to occur[37]. When seeded on an acellular amniotic membrane scaffold, UMSCs promote tissue regeneration and improve wound healing outcomes. This scaffold not only confers anti-adhesive, bacteriostatic, and epithelialization properties, but also attenuates the wound pain reported by patients[37]. In refining the applications for UMSCs, it is necessary to develop more efficient techniques for cell isolation, culture, and expansion. Lastly, it is essential to monitor patients for the possibility of tumor formation[27].

Embryonic stem cells

Embryonic stem cells (ESCs) constitute the latest and perhaps most up-and-coming category in stem cell therapy development. ESCs are pluripotent stem cells derived from the blastocyst stage of embryos and, given the correct conditions, are capable of differentiating into any cell in all three germ cell layers[42]. These ESC-derived cell lineages include hematopoietic stem cells, differentiated T cells, and epidermal cells. Characteristic cell surface markers include octamer-binding transcription factor 4 (Oct-4) and stage specific embryonic antigens (SSEAs). Because of their pluripotent nature, ESCs have the unique potential to serve as a therapeutic modality in the treatment of multiple disease processes, including chronic wounds. Preclinical studies have demonstrated that ESCs can differentiate into fully functioning human basal keratinocytes, which can further develop into stratified epidermis[102]. *In vivo* studies in mice have also shown that when compared to endothelial progenitor cells derived from cord blood, human ESCs exhibit improved dermal regeneration and re-epithelialization in chronic wounds.

The clinical use of ESCs is, however, limited by the ethical controversy surrounding their procurement. ESC therapy has been widely debated because currently, human ESCs cannot be obtained with causing significant damage to the human embryo. Proponents of ESC therapy maintain that this treatment will advance medical science

and that the politicization of scientific research stymies this progress[103]. On the other hand, induced pluripotent stem cells (iPSCs) offer an effective alternative to ESC therapy that demonstrate *in vitro* and *in vivo* potential in wound healing while circumventing the ethical concerns of ESCs. These iPSCs can be generated *via* programming of differentiated adult keratinocytes with embryonic-like stem cell properties. The process of reprogramming keratinocytes involves retroviral transduction of essential transcription factors, such as c-Myc, Klf4, Oct-3/4, and Sox2[48]. Nonetheless, the use of either iPSC or ESC therapy will require the medical community to address their respective safety concerns. Despite their associated risk for tumorigenesis and teratoma formation, these cells offer a promising option for the treatment of chronic wounds because of their ability to differentiate into all three germ cell layers[104].

DISCUSSION

The role of stem cell therapies in the treatment of chronic, non-healing wounds is continuously being refined within the scope of tissue engineering. Stem cells promote restoration of impaired signaling pathways for growth factors, delivery of important cytokines and chemokines, induction of vascularization and innervation, and more precise control of the inflammatory processes underlying chronic wounds[105]. Considerations with therapy usage involve donor variables, such as site and cell availability, patient age, and patient sex, as well as risks for diminished stem cell efficacy associated with repetitive culturing and possible malignant transformation. Nonetheless, stem cell therapy can be optimized extensively *via* a wide range of co-delivery techniques in the form of scaffolds, hydrogels, and other carriers with or without wound healing additives.

As with any systematic evaluation of the literature, there are some important limitations to consider with this review. Compared to preliminary, preclinical work, there are fewer clinical studies exploring the full scope of stem cell therapies for the treatment of chronic wounds. Given the lack of clear guidelines regarding therapy use for certain patient demographics, underlying health statuses, and presence of comorbid diseases, it is difficult to generalize the applicability of one therapy to another chronic wound etiology that has not been previously tested. Therapeutic success is determined on a case-by-case basis.

However, there are ongoing investigative efforts to improve understanding of stem cell therapies. Recently, the use of electrospun fiber scaffolds for stem cell therapy culture has been explored with the goal of enhancing cell proliferation and differentiation. This technique additionally opens up new possibilities for controlled fiber morphology and structure to generate layered skin substitute dressings[32]. Though clinical studies and trials have supplemented our knowledge of stem cell therapy usage for chronic, non-healing wounds, further studies are needed in order to more comprehensively examine the breadth of this therapeutic modality and more closely personalize wound care regimens for each individual patient. As these treatments become more advanced by optimizing wound healing outcomes while minimizing donor morbidity, stem cell-based therapy is likely to establish itself as a mainstay of chronic wound care and management.

ARTICLE HIGHLIGHTS

Research background

Chronic wounds are defined as those that do not heal within a period of 3 mo, resulting in significant patient morbidity and healthcare burden. Due to local tissue hypoxia, bacterial colonization, ischemia-reperfusion injury, and diminished stem cell populations, these wounds do not progress through the normal wound healing phases. Further, non-healing wounds are attributable to a host of etiologies, including arterial disease, diabetes, vasculitis, venous valve insufficiency, irradiation, and malignancy. Their complex pathophysiology poses a formidable treatment challenge, and presently, there are ineffective techniques to facilitate wound closure and improved patient symptomatology. Stem cell therapies have therefore emerged as a unique therapeutic approach to modulate the chronic wound environment in favor of healing. In this systematic review, we evaluate literature over the past two decades to ascertain clinical findings associated with stem cell therapies for treating chronic

wounds.

Research motivation

While adipose-derived stem cells (ADSCs) and bone marrow-derived stem cells (BMMSCs) have been tested the most frequently in clinical settings, it is unclear how other emerging stem cell therapy types function in healing chronic wounds. It is critical that we comprehensively consider a variety of stem cell therapies for the treatment of a diverse scope of non-healing wounds in order to provide maximal clinical benefit to wound care specialists and providers.

Research objectives

To investigate the scope of a variety of stem cell therapies, including adipose-derived stem cells (ADSCs), bone marrow-derived stem cells (BMMSCs), bone marrow-derived mononuclear cells (BMMNCs), epidermally-derived mesenchymal stem cells (EMSCs), fibroblast stem cells (FSCs), keratinocyte stem cells (KSCs), placental mesenchymal stem cells (PMSCs), umbilical cord mesenchymal stem cells (UMSCs), and embryonic stem cells (ESCs), for the treatment of chronic, non-healing wounds.

Research methods

We performed a systematic review of the literature according to the 2009 PRISMA guidelines. Five authors conducted a search in five databases (PubMed, EMBASE, Cochrane Library, Web of Science, and Scopus) to identify relevant publications, articles, and abstracts reporting clinical stem cell therapy use for chronic wounds from the years 2000 to 2019.

Research results

A total of 43 studies were included in this review. The studies reported that ADSCs and BMMSCs have been tested in the widest scope of clinical applications, including the treatment of severe radiation-associated wounds, venous leg ulcers, chronic fistulae, chronic diabetic ulcers, and advanced pressure ulcers from spinal cord injury. Enhanced testing of other stem cell therapy types has provided informative guidelines for therapy optimization, including seeding stem cells into artificial dermal, wound matrix, and hydrogel scaffolds for improved cell survival and proliferation in the wound beds. FSCs and KSCs can be delivered with additives, including fibrin, to strengthen cell properties of adherence, migration, and epithelial monolayer formation. Improved wound healing with each of the stem cell therapy types was determined on the basis of histological and functional parameters. No studies reported significant complications with clinical use of any of the investigated therapies.

Research conclusions

Stem cells promote healing of chronic wounds by restoring impaired signaling pathways for growth factors, ensuring delivery of important cytokines and chemokines, inducing vascularization and innervation, and modulating inflammatory processes. Selecting optimal therapy for various wounds is contingent on patient variables, including age, sex, and stem cell donor site, as well as processing variables, such as culturing after cell harvest and potential for malignant transformation. Additional clinical studies are required to replicate the strength of the literature findings for ADSCs and BMMSCs and substantiate use of a wider scope of stem cell therapies for treating non-healing wounds.

Research perspectives

There is limited clinical evidence examining the use of EMSCs, FSCs, KSCs, PMSCs, UMSCs, and ESCs for the treatment of chronic wounds, though these stem cells have demonstrated potential in preclinical *in vitro* and *in vivo* work. Further studies exploring the use of these therapies for clinically diverse patient wounds would be highly informative. In addition to aforementioned artificial dermal, wound matrix, and hydrogel scaffolds, there is ongoing development with newer cell delivery constructs, such as electrospun fiber scaffolds to facilitate creation of layered skin substitute dressings. As the etiology of chronic wounds varies from patient to patient, it is necessary to personalize therapeutic approaches. The goal of future investigations will be to further realize improved patient wound closure and histologic markers of wound healing, as well as decreased pain, disfigurement, and healthcare system burden.

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SYSTEMATIC REVIEWS

Application and prospect of adipose stem cell transplantation in treating lymphedema

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Abstract

BACKGROUND

Lymphedema is a chronic, debilitating and incurable disease that affects 0.13%-2% of the global population. Emerging evidence indicates that adipose-derived stem cells (ADSCs) might serve as suitable seed cells for lymphatic tissue engineering and lymphedema therapy.

AIM

To summarize applications of ADSCs for treating lymphedema in both animal studies and clinical trials.

METHODS

A systematic search was performed on four databases – PubMed, Clinicaltrials.gov, the evidence-based Cochrane Library, and OVID – using the following search string: ("lymphedema" or "lymphoedema" or "lymphangiogenesis") and ("adipose-derived stem cells" or "adipose-derived stromal cells" or "adipose-derived regenerative cells"). A manual search was performed by skimming the references of relevant studies. Animal studies and clinical trials using adipose-derived cells for the treatment of any kind of lymphedema were included.

RESULTS

A total of eight research articles published before November 2019 were included for this analysis. Five articles focused on animal studies and another three focused on clinical trials. ADSC transplantation therapy was demonstrated to be effective against lymphedema in all studies. The animal studies found that coadministration of ADSCs and controlled-release vascular endothelial growth factor-C or platelet-rich plasma could improve the effectiveness of ADSC therapy. Three sequential clinical trials were conducted on breast cancer-related

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lymphedema patients, and all showed favorable results.

CONCLUSION
ADSC-based therapy is a promising option for treating lymphedema. Large-scale, multicenter randomized controlled trials are needed to develop more effective and durable therapeutic strategies.

Key Words: Lymphedema; Adipose-derived stem cells; Animal model; Clinical trial; Vascular endothelial growth factor-C; Lymphangiogenesis

Core tip: Lymphedema is a growing global health problem. Adipose-derived stem cells might serve as suitable seed cells for tissue engineering of lymphatic vessels *in vitro* and *in vivo*. A systematic search of publications on the application of adipose-derived stem cells in the treatment of lymphedema identified five animal studies and three clinical trials. All eight studies showed improvement of lymphedema after treatment with adipose-derived stem cells. Animal studies conducted with acute lymphedema mouse models provided data for finding the proper dose and methods of administration. Clinical trials were conducted on breast cancer-related lymphedema patients and are important references for further application.

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INTRODUCTION

As the second circulatory system of the body, the lymphatic system functions to transport tissue fluid in the interstitial space back to the venous circulation system and maintain fluid homeostasis. Hypoplasia or dysfunction of lymphatic vasculature may result in "lymphedema"[1], which is characterized by retention of lymphatic fluid in the interstitial space, leading to a series of pathological changes, including tissue swelling, chronic inflammation, lipid deposition, and tissue fibrosis[2].

There are two types of lymphedema according to etiology[3]. Primary lymphedema comes from developmental or congenital abnormalities of the lymphatic system resulting in dysfunctional lymphatics, which could be symptomatic at birth or more commonly in adolescence. Secondary lymphedema is more common and results from trauma, obstruction, surgery, or infection involving the lymphatic system. Up to 250 million people in developing countries suffer from lymphedema, with the parasitic disease filariasis as the most prevalent cause[4]; lymphadenectomy and radiation therapy for cancer treatment are usually the major causes of lymphedema in developed countries.

Chronic lymphedema affects 0.13%-2% of the global population[5]. It is estimated that one out of six patients undergoing treatment for a solid tumor will eventually develop lymphedema[6]. For patients with breast cancer, 24%-49% of those receiving mastectomy will develop upper extremity lymphedema[7]. The persistence of the disease, burden of treatment, and likelihood of progression press heavy medical and socioeconomic burdens onto patients. Therefore, development of effective therapies for lymphedema is of vital importance.

Lymphedema can be treated conservatively, surgically, or by a combination of them[2]. Conservative therapies consist of complex decongestive therapy, manual lymphatic drainage, and exercise[8]. Surgical therapies include liposuction, wedge resection, Charles procedure (radical excision for limb lymphedema), and lymphatic reconstruction or bypass techniques[9]. However, there is a lack of effective and feasible therapies which could radically cure lymphedema[10]. Therefore, elucidating the underlying pathophysiological mechanisms of lymphedema holds promise for the treatment of lymphedema.

Progenitor or stem cell-based therapies, which treat diseases through regeneration, have represented an alternative treatment method not only for lymphedema but for a wide spectrum of other diseases as well. Due to their abundant resources, easy access, pluripotent capacity, and harboring of few ethical and immunological issues, the adipose-derived stem cells (ADSCs) are considered as one of the most promising seed cell types for regenerative medicine[11-15]. In addition, ADSCs exhibit paracrine immunomodulatory and trophic effects in their local microenvironment. Emerging *in vitro* studies have investigated the possible mechanisms and benefit of ADSCs in the treatment of lymphedema, such as their capacity for differentiation into lymphatic endothelial cells (LECs)[16,17] and paracrine secretion of cytokines[18], chemokines[19] and exosomes[20], thereby promoting angiogenesis and modulating the immune response. Takeda *et al*[21] reported that, by secreting lymphangiogenic factors, ADSCs promote proliferation, migration, and tube formation of LECs *in vitro*. Deng *et al*[22] demonstrated that overexpression of Prox1 in human ADSCs (*via* lentiviral vectors) induces the differentiation of human ADSCs into stable lymphatic endothelial-like cells *in vitro* and that the differentiated cells form tube-like structures (as shown in tube formation assay). Yen *et al*[23] showed that ADSCs promote lymphangiogenesis under stimulation of vascular endothelial growth factor-C (VEGF-C), a key lymphangiogenic factor, or in response to inhibition of TGF-β1; moreover, stimulation of ADSCs with VEGF-C was found to markedly increase cellular proliferation and cellular survival after *in vivo* implantation and to induce the expression of podoplanin, a lymphangiogenic cell marker. Sun *et al*[19] reported that interleukin-7 enhanced the differentiation of ADSCs into LECs *via* AKT signaling pathways. Most recently, Saijo *et al*[24] revealed that paracrine effects of ADSCs promoted lymphangiogenesis in irradiated LECs. They reported that coculture with ADSCs and the use of ADSC-conditioned medium improved proliferation, migration, and tube formation of nonirradiated LECs. Furthermore, they demonstrated that irradiated ADSCs can exert similar alleviative effects to irradiated human dermal LECs[24].

Collectively, these research findings have suggested that ADSCs might serve as suitable seed cells for lymphatic tissue engineering and secondary lymphedema therapy. The aim of this review is to systematically summarize the application of ADSCs for lymphedema treatment in animal studies and in clinical trials. In addition, the future perspectives of ADSCs in lymphedema therapy are discussed.

MATERIALS AND METHODS

A systematic search was performed on four databases (PubMed, Clinicaltrials.gov, the evidence-based Cochrane library, and OVID) using the following search string: ("lymphedema" or "lymphoedema" or "lymphangiogenesis") and ("adipose-derived stem cells" or "adipose-derived stromal cells" or "adipose-derived regenerative cells"). After duplicate removal, all studies were screened based on title and abstract. Furthermore, full-text versions of included studies were read for further evaluation. A manual search was also performed by skimming the references of included studies. The search process is presented in Figure 1.

The inclusion criteria were animal studies and clinical trials using adipose-derived cells for treatment of any kind of lymphedema, which had been published no later than November 2019. The exclusion criteria were non-English language, reviews, or *in vitro* studies. For animal studies, data retrieved were year of publication, first author, type of animal models, cell type used (freshly isolated or culture-expanded as well as autologous or allogeneic), cell dosage, cell characterization (cell count/viability, surface marker analysis, *etc*.), means and routes of transplantation (alone or in combination with growth factors, scaffolds, *etc*.), assessment types, and results. For clinical trials, data retrieved were year of publication, country of origin, disease treated, study design (randomized controlled trial, nonrandomized study, or case series/pilot study), number of participants, cell type used (freshly isolated or culture-expanded as well as autologous or allogeneic), cell dosage, cell characterization (cell count/viability, surface marker analysis, *etc*.), means and routes of transplantation (alone or in combination with growth factors, scaffolds, *etc*.), assessment types, and outcomes.

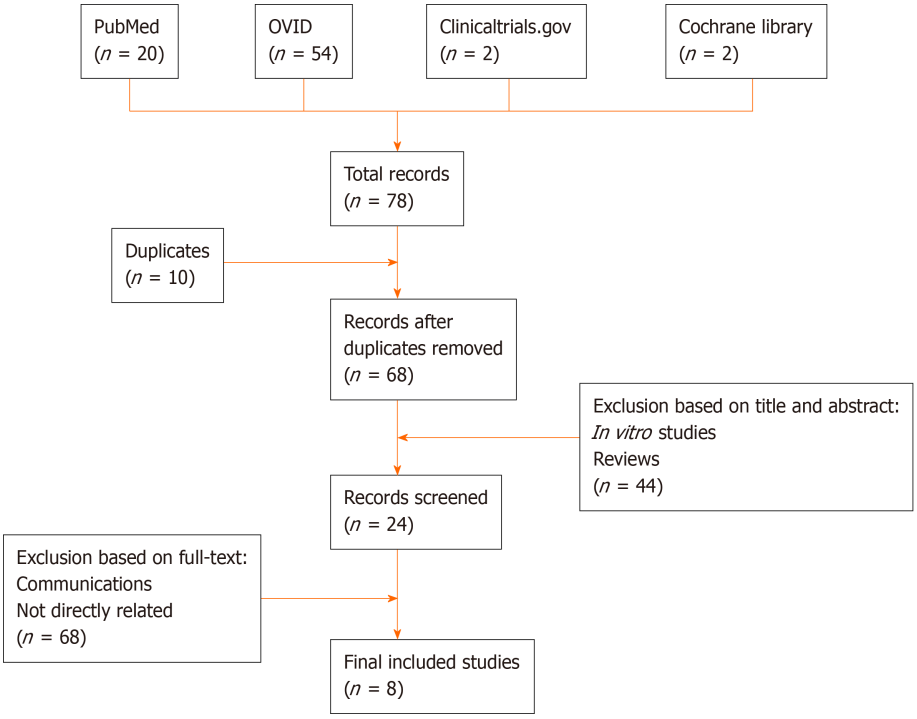

Figure 1 Search process of articles regarding adipose-derived stem cell-based treatment of lymphedema studied in animal models and clinical trials.

RESULTS

A total of eight research articles published before November 2019 met the inclusion criteria for this analysis. These included five articles focused on animal studies (Table 1) and three focused on clinical trials (Table 2).

Animal studies

A total of five animal studies were included in the analysis. Commonly-used animal models were mouse hindlimb or tail model of lymphedema. The surgical procedures were circumferential incision with or without radiation. The number of cells used for injection varied from 10^4 to 10^{10} each time. Various treatments were used in combination with cell injection, including controlled-release VEGF-C, platelet-rich plasma (PRP), or vascularized lymph node transfer. Treatment outcomes were evaluated by circumference, dermal edema depth, imaging (lymphangiography and photodynamic dye), and histochemical and immunohistochemical staining (for CD31, LYVE1, and VEGF receptor).

Hwang *et al*[25] established a mouse hindlimb model of lymphedema by electrocauterizing the lymph vessels in the thigh following a circumferential incision. *In vivo* study demonstrated that combination of human (h) ADSCs and VEGF-C hydrogel markedly alleviated dermal edema and increased lymphatic vessel density when compared with results achieved with hADSCs or VEGF-C hydrogel alone at various post-treatment time points (from 3-4 d to 4 wk post-treatment). In addition, the authors demonstrated the existence of hADSCs in all of the implantation sites in the hADSC/VEGF-C group with LECs phenotype. Their results also suggested that, in conjunction with hADSCs, VEGF-C-containing hydrogels could serve as suitable delivery vectors to improve lymphangiogenesis.

Shimizu *et al*[26] reported on the establishment of a mouse tail model of lymphedema. They made a 2 mm-wide circumferential excision on the skin 10 mm distal to the tail base and excluded a 4 mm^2 dermal flap at the ventral side. They indicated that local injection of 2×10^6 freshly isolated autologous ADSCs could reduce lymphedema and accelerate lymphangiogenesis at the congestive lymphedema region. The authors also revealed that ADSCs could release VEGF-C to stimulate lymphangiogenesis and recruit bone marrow-derived M2 macrophages to serve as lymphatic endothelial progenitor cells.

Ackermann *et al*[27] assessed the effects of PRP and adipose stem cells on angiogenesis, microcirculation, lymphangiogenesis, microvascular architecture, and

Table 1 Adipose-derived stem cell-based treatment of lymphedema in animal studies

Year	Ref.	Animal	Location and methods	Groups	Cell type used for characterization and number	Implantation methods	Assessment	Results
2011	Hwang *et al*[25]	6-8-wk-old female BALB/c mice	Hindlimb, Circumferential incision and electrocautery	5 groups (n = 5 for each group): Normal, control, hADSCs, VEGF-C hydrogel, hADSCs/VEGF-C hydrogel	PKH-26-labeled hADSCs N/A	In combination with VEGF-C gelatin hydrogel subcutaneous injection	Dermal edema depth measurement using Vernier calipers H&E staining IFC (LYVE-1) for lymphatic vessel intensity	Co-administration of hADSCs and VEGF-C decreased dermal edema depth and increased lymphatic vessel intensity
2012	Shimizu *et al*[26]	7-8-wk-old male C57BL/6J mice	Tail 2 mm-wide circumferential annulus of the skin excision at 10 mm distal to the tail base, excluding a 4 mm^2 dermal flap located at the ventral side	3 groups (n = 12 for each group): Sham, PBS, and freshly isolated ADRCs	Freshly isolated ADRCs 2 × 10^6	Local injection	Tail thickness measurement IHC analysis (LVYE-1) IFC staining (LYVE-1, CD11-b, CD163)	Local injection of ADRCs significantly reduced lymphedema ADRC implantation accelerated lymphangiogenesis ADRC implantation enhanced recruitment of M2 macrophages, to serve as lymphatic endothelial progenitor cells
2015	Ackermann *et al*[27]	10-wk-old male C57BL/6J mice	Tail Circumferential 5 mm-wide full thickness excision at a 10 mm distance from the base of the tail	3 groups: Saline, PRP, and ASC	Allogenic 3 passages FACS analyzed (CD31−/CD45−/CD29+/CD90+ cells	Injection	Angiogenesis (anti-CD31 staining) Laser Doppler imaging for microcirculation Lymphangiogenesis (anti-LYVE1 staining) Corrosion casting for microvascular architecture Digital planimetry for wound healing	Wounds treated by PRP and ASC healed faster and showed a significantly increased epithelialization Application of PRP induced a significantly increased lymphangiogenesis, while application of ASC did not induce any significant change, in this regard.
2015	Yoshida *et al*[28]	Male C57BL/6J mice	Right hindlimb, 30-gray x irradiation, surgical lymph node dissection, and 2-mm gap creation	4 groups (n = 20 for each group with different cell numbers)	Allogenic up to 5 passages 0, 10^6, 10^5, 10^4	Subcutaneous injection	Circumference measurement Near-infrared video camera for lymphatic flow assessment IHC for quantitation of lymphatic vessels (LYVE, VEGF-C, VEGFR, and EGFP) XY chromosome FISH analysis	Number of lymphatic vessels significantly increased at 2 wk No direct detection of ADSCs involving lymphangiogenesis by EGFP at 2 wk or chromosome FISH at 2 wk and 4 wk

2017	Hayashida *et al*[29]	10-wk-old male C57BL/6J mice	Left hindlimb, 30-Gy X-ray irradiation, surgical lymph node dissection, and 5-mm gap creation	4 groups (n = 5 for each group): Control, VLNT, ADSCs, VLNT-plus/ADSCs-plus	Allogenic 1-3 passages at 10^4	VLNT, subcutaneously	Near-infrared video camera for lymphatic flow assessment Water-displacement plethysmometer for hind-paw volumetry test IHC for tissue quantification of lymphatic vessels (LYVE-1, VEGF-C, and VEGF-R3) B16 mouse melanoma cells for functional analysis of lymphatic vessels and nodes	Increased number of lymphatic vessels Induced lymphatic flow drainage to the circulatory system

ADRCs: Adipose-derived regenerative cells; ADSCs: Adipose-derived stem cells; ASC: Adipose stem cells; EGFP: Enhanced green fluorescent protein; FISH: Fluorescent *in situ* hybridization; H&E: Hematoxylin and eosin; hADSCs: Human adipose-derived stem cells; IFC: Immunofluorescence; IHC: Immunohistochemistry; N/A: Not applicable; PBS: Phosphate-buffered saline; VEGF-C: Vascular endothelial growth factor-C; VEGF-R3: Vascular endothelial growth factor-receptor 3; VLNT: Vascularized lymph node transfer.

wound healing in a mouse tail lymphedema model. They found that treatment with PRP and adipose stem cells could accelerate wound healing and increase epithelialization. PRP application induced a remarkably improved lymphangiogenesis. Ultimately, the authors drew the conclusion that PRP and adipose stem cells can affect lymphangiogenesis and lymphedema development.

Yoshida *et al*[28] reported on the establishment of a mouse right hindlimb secondary lymphedema model, using 30 Gy X-ray irradiation 7 d before surgery to create a circumferential incision and a gap of approximately 2 mm, left open. Circumferential measurement, lymphatic flow assessment, and quantification of lymphatic vessels demonstrated that at 14 d post-treatment of local injection with 10^6 ADSCs, 10^5 ADSCs, or 10^4 ADSCs, the numbers of lymphatic vessels were significantly increased in the transplanted groups; the authors concluded that in secondary lymphedema, ADSCs could increase collecting vessels and reconstruct the lymphatic vascular network. A more recent study from the same research group[29] demonstrated that local implantation of 10^4 ADSCs in combination with vascularized lymph node transfer decreased tissue volume, increased lymphatic vessels density, and improved the lymphatic function in a slightly different mouse hindlimb model (left *vs* right hindlimb, 5 mm- *vs* 2 mm-wide gap, left open).

Clinical trials

A total of three clinical trials were included in the analysis. All these studies were conducted by the same research group in Denmark[25-27], focusing on upper limb and breast cancer-related lymphedema (BCRL), using freshly isolated autologous ADSCs

Table 2 Adipose-derived stem cell treatment of lymphedema in clinical trials

Year	First author	Study type	Patient number, test/control	Edema location	Cell type	Cell number	Injection method, location/depth	Follow-up inmo assessment	Results
2016	Toyserkani *et al*[30]	Pilot study	1/0	Upper limb	Freshly isolated autologous ADSCs plus fat graft	4.07×10^7 plus 10 mL lipoaspirate	Axillary region (8 points)	4 mo *In vitro* cell characterization (cell count, viability, and surface marker)	Great reduction in symptoms of arm heaviness and tension, reduction in need for compression therapy
								Circumference measurements	Volume reduction in affected arm
								Dual-energy X-ray absorptiometry scans Adverse events	No postoperative complications or adverse events
2017	Toyserkani *et al*[31]	Pilot study	10/0	Upper limb	Freshly isolated autologous ADSCs plus fat graft	5×10^7 plus 30 mL lipoaspirate	Axilla (8 points)	6 mo *In vitro* cell characterization (cell count, viability, and surface marker)	A small volume reduction but not significant
								Circumference measurements	Patient-reported outcomes improved significantly over time
								Dual-energy X-ray absorptiometry scans	Half of the patients reduced their use of conservative management
								Patient-reported outcome and safety questionnaire assessment	No noteworthy serious adverse events
2019	Toyserkani *et al*[32]	Pilot study	10/0	Upper limb	Freshly isolated autologous ADSCs plus fat graft	5×10^7 plus 30 mL lipoaspirate	Axilla (8 points)	12 mo *In vitro* cell characterization (cell count, viability, and surface marker)	No significant change in volume
								Circumference measurements	Patient-reported outcomes improved significantly over time
								Dual-energy X-ray absorptiometry scans	Five patients reduced their use of conservative management
								Patient-reported outcome and safety questionnaire assessment	Quantitative lymphoscintigraphy showed no improvement on the lymphedema-affected arms
								Lymphoscintigraphy changes	ADRCs were well-tolerated and only minor transient adverse events related to liposuction were noted

ADRCs: Adipose-derived regenerative cells; ADSCs: Adipose-derived stem cells.

with fat grafting. The follow-up period ranged from 4 mo to 1 year.

In 2016, Toyserkani *et al*[30] reported a pilot study using ADSC-assisted lipotransfer to treat lymphedema. A 48-year-old female patient developed BCRL after lymphadenectomy and radiation therapy. A total of 4.07×10^7 freshly isolated autologous ADSCs were administered, along with 10 mL of lipoaspirate (for fat-grafting), to the axillary region. At 4 mo post-treatment, the authors noted that the daily symptoms of arm heaviness and tension were greatly relieved, the needs for compression therapy were reduced, and the volume of the affected arm was decreased without postoperative complications or adverse events.

Furthermore, the authors had also performed a larger study to validate the feasibility and safety of this procedure in 2007[31], which was registered at

Clinicaltrials.gov under the identifier NCT02592213 at phase 2 stage. In this pilot study, ten BCRL patients were included. Combined with a scar-releasing fat graft (about 30 mL) procedure, approximately 5 × 10^7 freshly isolated adipose-derived regenerative cells (ADRCs) were injected into the axillary region. During a 6-mo follow-up period, there was a small but not significant volume reduction. Five of the patients showed a reduced need for conservative treatment, and patient-reported follows-up improved significantly over time. Slight, temporary adverse events were observed, but were more likely caused by liposuction procedures rather than ADRC injection. Ultimately, the ADRCs were deemed as well-tolerated.

In 2019, the results of lymphoscintigraphic evaluation with 1-year follow-up were reported[32]. Consistent with the results of the 6-mo follow-up, ADRC injection improved lymphedema, as revealed by patient-reported outcomes without serious adverse events. However, there was no improvement in lymphoscintigraphic evaluation and no change in arm volume after the ADRC treatment. Now, this research group is recruiting patients for a randomized phase 3 trial, which is registered at Clinicaltrials.gov under the identifier NCT03776721. This study started December 17, 2018 and the estimated study completion date is September 1, 2021, designed to recruit 80 participants with a parallel assignment to evaluate the efficacy and safety of implantation of freshly isolated adipose-derived stromal cells in combination with fat grafting at the affected axillary region.

DISCUSSION

Lymphedema, characterized by tissue swelling, lipid deposition, and fibrosis due to excess accumulation of interstitial fluid and inadequate lymphatic drainage, affects 0.13%-2% of the global population[5] and remains a chronic, debilitating and incurable disease. Stem cell-based regenerative medicine has shown great promise for refractory diseases, such as inflammatory bowel diseases[33], heart failure[34], osteoarthritis[35], rheumatoid arthritis[36], and graft-versus-host disease[37]. With the properties of self-renewal, multipotential differentiation, paracrine, immunomodulatory, and trophic effects, and low immunogenicity alongside their practical advantages, ADSCs have become one of the most promising candidates for regenerative medicine.

ADSCs are isolated from the aqueous fraction – known as the stromal vascular fraction (SVF) – by means of enzymatic digestion of lipoaspirate (liposuction product)[38,39]. As the major source of ADSCs, SVF is a heterogeneous cell group composed of ADSCs, endothelial precursor cells, endothelial cells, macrophages, smooth muscle cells, lymphocytes, pericytes, and pre-adipocytes derived from fat tissue. Recent advances have shown the role and efficacy of SVF and ADSCs in improvement of tissue regeneration, especially in plastic reconstruction[40], such as breast reconstruction[41,42], wound healing[43,44], scars[45,46], and soft tissue defects[47]. The SVF is more easily obtained, regardless of cell separation and culture conditions. Therefore, therapeutic cellular products are obtained immediately with minimal contact with reagents, making it not only technically easier but also relatively safer. At the same time, the unique heterogeneous cell components of SVF may achieve better treatment results in comparative animal studies. It is worth noting that SVF might be suitable for autologous therapy only, due to the existence of various cell types that might cause immunological rejection[48], whereas ADSCs are useful in both allogenic therapy and autologous therapy.

As mentioned in the first part of this review, emerging *in vitro* studies have already definitively demonstrated the advantage of ADSCs in lymphangiogenesis and treatment of lymphedema. All five animal studies included in this systematic review were performed on mouse models and the ADSCs were injected immediately or shortly after lymphedema induction. These studies examined the effect of ADSCs on acute lymphedema and exhibited improvement at 4-6 wk. However, the anatomy, physiology, and healing capacity are quite different between mice and human beings, bringing up the need for larger animal models in order to simulate chronic lymphedema in human beings more accurately. Further preclinical studies with better animal models are needed to determine whether ADSC-based therapies could fulfil expectations and be extrapolated to clinical use in patients. As is commonly known, to observe lymphatics in mice, it is usually necessary to visualize the blue dye-stained lymphatic vessels in dissected tissues or tissue sectioning for immunohistological staining for the lymphatic markers, such as VEGFR-3, PROX1, and LYVE1, to quantitatively identify lymphatic vessel intensity and lymphangiogenesis. Advances in near-infrared fluoroscopy lymphatic imaging[49], magnetic resonance imaging agents

based on nanotechnology, and gene reporter technologies have paved roads for depicting functions of lymphatic vasculature. Though not all lymphatic imaging methods are suitable for both clinical and preclinical trials, their effective combination will provide new tools for lymphedema translational medicine[50].

Therapy of lymphedema has benefitted substantially from the most recent advances in lymphatic vessel engineering and regenerative medicine, including cell-seeded scaffolds for vessel reconstruction, implantation of stem cells, prolymphangiogenic growth factors, or a combination of these technologies. As mentioned above, when implanted into the injury site in a lymphedema mouse model, the gelatin hydrogel containing controlled-release VEGF-C could encapsulate hADSCs, increase vessel density, and improve dermal edema, leading to efficient application in lymphatic vessel regeneration[25]. It has been reported that SVFs and ADSCs could improve wound healing when used alone or in combination with PRP and hyaluronic acid, specifically in healing of lower-extremity soft- and hard-tissue wounds[43] and in severe hidradenitis suppurativa wounds[47]. Many other studies confirmed that SVFs and ADSCs can improve wound healing when used alone or in combination with PRP and fat graft[41,45,51,52]. Considering the similar biological processes and biomolecular pathways shared between wound healing and lymphedema, such as inflammation and angiogenesis, the efforts towards establishing ADSCs-based therapy for lymphedema will benefit from these studies. Certainly, the research efforts towards development of cell isolation and expansion methods and of three-dimensional scaffolds[53], nanoparticles, and targeted delivery will help to optimize and maximize the efficacy of ADSC-assisted therapy. Standardized platforms with minimized manipulations and maximized efficacy will facilitate both up-scaling and the ease of clinical translation.

Over the past few years, concerns regarding the safety of stem cell application have also been raised. Indeed, Toyserkani *et al*[54] performed a systematic review, including over 1400 patients who received ADSC treatment and were followed for 4 wk to 3 years, in order to assess the safety of ADSC therapy, concentrating on the risks of thromboembolical, immunological, and oncological safety concerns. They reported that the observed adverse outcomes were relevant to liposuction, injuries from implantation, or the underlying condition, rather than the ADSC therapy itself. Although the ADSC therapy has shown a favorable safety performance, more reliable and rigorous safety assessment approaches are still encouraged for further study and clinical practice.

In conclusion, the results of *in vitro* studies, animal models, and clinical trials characterize ADSC-based treatment as a promising option and one that can be used within biologically rational and controllable environments for the treatment of lymphedema. However, further investigations in larger animal models and larger-scale, multicenter randomized clinical trials with more reliable and rigorous safety assessments are needed to develop more effective and durable therapeutic strategies.

ARTICLE HIGHLIGHTS

Research background

Lymphedema is a chronic, debilitating and incurable disease that affects 0.13%-2% of the global population. Emerging evidence indicates that adipose-derived stem cells (ADSCs) might serve as suitable seed cells for lymphatic tissue engineering and lymphedema therapy. Here, we appraise the *in vivo* evidence for the application of ADSCs for lymphedema treatments.

Research motivation

Emerging research findings have suggested that ADSCs might serve as suitable seed cells for tissue engineering of lymphatic vessels *in vitro* and potentially in the treatment of secondary lymphedema *in vivo*. It is critical that we systematically summarize the application of ADSCs for lymphedema treatments in animal studies and in clinical trials and discuss the future perspectives.

Research objectives

The main objectives of this review are to systematically summarize the application of ADSCs for lymphedema treatments as shown in animal studies and clinical trials. In addition, the future perspectives of ADSCs in lymphedema therapy are discussed.

Research methods

A systematic search was performed on four databases – PubMed, Clinicaltrials.gov, the evidence-based Cochrane library, and OVID – using the following search string: ("lymphedema" or "lymphoedema" or "lymphangiogenesis") and ("adipose-derived stem cells" or "adipose-derived stromal cells" or "adipose-derived regenerative cells"). A manual search was performed by skimming the references of relevant studies. Animal studies and clinical trials using adipose-derived cells for the treatment of any kind of lymphedema were included.

Research results

A total of eight research articles published before November 2019 were included for this analysis. Five articles focused on animal studies and another three focused on clinical trials. ADSC transplantation therapy was demonstrated to be effective against lymphedema in all studies. The animal studies found that coadministration of ADSCs and controlled-release vascular endothelial growth factor-C or platelet-rich plasma could improve the effectiveness of ADSC therapy. Three sequential clinical trials were conducted on breast cancer-related lymphedema patients, and all showed favorable results.

Research conclusions

The results of *in vitro* studies, animal models, and clinical trials characterize ADSC-based treatment as a promising option and one that can be used within biologically rational and controllable environments for the treatment of lymphedema.

Research perspectives

Further preclinical studies in larger animal models and large-scale, multicenter randomized controlled clinical trials with more reliable and rigorous safety assessments are needed to develop more effective and durable therapeutic strategies. Advances in lymphatic imaging methods will provide opportunities for lymphedema translational medicine as well.

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SYSTEMATIC REVIEWS

Mesenchymal stem cells and mesenchymal stem cell-derived extracellular vesicles: Potential roles in rheumatic diseases

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Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) have been widely investigated in rheumatic disease due to their immunomodulatory and regenerative properties. Recently, mounting studies have implicated the therapeutic potency of MSCs mostly due to the bioactive factors they produce. Extracellular vesicles (EVs) derived from MSCs have been identified as a promising cell-free therapy due to low immunogenicity. Rheumatic disease, primarily including rheumatoid arthritis and osteoarthritis, is a group of diseases in which immune dysregulation and chronic progressive inflammation lead to irreversible joint damage. Targeting MSCs and MSC-derived EVs may be a more effective and promising therapeutic strategy for rheumatic diseases.

AIM

To evaluate the potential therapeutic effectiveness of MSCs and EVs generated from MSCs in rheumatic diseases.

METHODS

PubMed was searched for the relevant literature using corresponding search

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terms alone or in combination. Papers published in English language from January 1999 to February 2020 were considered. Preliminary screening of papers concerning analysis of "immunomodulatory function" or "regenerative function" by scrutinizing the titles and abstracts of the literature, excluded the papers not related to the subject of the article. Some other related studies were obtained by manually retrieving the reference lists of papers that comply with the selection criteria, and these studies were screened to meet the final selection and exclusion criteria.

RESULTS

Eighty-six papers were ultimately selected for analysis. After analysis of the literature, it was found that both MSCs and EVs generated from MSCs have great potential in multiple rheumatic diseases, such as rheumatoid arthritis and osteoarthritis, in repair and regeneration of tissues, inhibition of inflammatory response, and regulation of body immunity *via* promoting chondrogenesis, regulating innate and adaptive immune cells, and regulating the secretion of inflammatory factors. But EVs from MSCs exhibit much more advantages over MSCs, which may represent another promising cell-free restorative strategy. Targeting MSCs and MSC-derived EVs may be a more efficient treatment for patients with rheumatic diseases.

CONCLUSION

The enormous potential of MSCs and EVs from MSCs in immunomodulation and tissue regeneration offers a new idea for the treatment of rheumatism. However, more in-depth exploration is needed before their clinical application.

Key Words: Mesenchymal stem cell; Extracellular vesicle; Autoimmunity; Inflammation; Rheumatoid arthritis; Osteoarthritis

Core tip: Mesenchymal stem cells (MSCs) and MSC-derived extracellular vesicles (EVs) have long been thought to possess considerable immunomodulatory and regenerative potential. Rheumatic disease is a group of diseases marked by immune dysregulation and chronic progressive inflammation. Targeting MSCs and MSC-derived EVs may be a more efficient treatment for rheumatic diseases. However, before their application in the clinical treatment, a large number of preclinical studies and clinical studies are required to thoroughly assess their safety and efficiency. This work summarizes current advances and offers a strong basis for the next study of MSCs and MSC-derived EVs in this field.

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INTRODUCTION

Rheumatic disease is a group of diseases with high morbidity over the world that can affect the musculoskeletal system, leading to arthritis, joint damage, and joint disability[1,2]. Rheumatoid arthritis (RA) and osteoarthritis (OA) are the two most prevalent rheumatic diseases with arthritis worldwide[1,2]. RA is a common systemic autoimmune disorder characterized by hyperplasia of the synovial membrane, infiltration of inflammatory cells, bone and cartilage progressive damage, and multiple organ involvement[3]. Uncontrolled and progressive inflammation and joint damage make RA patients have irreversible joint deformity and decreased life quality. Although disease-modifying anti-rheumatic drugs and nonsteroidal anti-inflammatory drugs have been routinely applied in the clinic to prevent or delay the progression of the disease, the effective therapy to cure RA patients is still absent. Another prevalent joint condition in the elderly is OA, a disease marked by irreversible degeneration of

multi-articular cartilage, changes of the underlying bone structure, synovitis, and osteophyte formation[4]. Various pro-inflammatory mediators are deemed to participate in the pathogenesis of OA, such as matrix metalloproteinases (MMPs), tumor necrosis factor-α, and interleukin (IL)-6. The contribution of imbalance between anabolism and catabolism in the joint as well as the load of mechanical stress to OA has been shown in a previous study[5]. Despite advances in the treatment of rheumatic diseases, their pathogenesis remains largely unknown.

The immune regulatory and regenerative effects of mesenchymal stem cells (MSCs) provide new insight into the treatment of rheumatic diseases. It has been demonstrated that MSCs can be used to treat RA and OA by regulating both innate and adaptive immune cells[6,7]. MSCs can suppress the multiplication and development of T cells and B cells, induce more regulatory T cells (Tregs), promote the polarization of M2 macrophages, impair the function of dendritic cells (DCs), as well as decrease the maturation and cytotoxicity of natural killer (NK) cells[8] (Figure 1). In addition, it has been demonstrated that the predominant mechanism by which MSCs exert their effects is producing a large variety of paracrine, rather than contact-dependent, mediators[9], although MSCs can work either directly or indirectly. These mediators include growth factors, cytokines, chemokines and so forth[10], among which extracellular vesicle (EV) is one of the most important kind which can mimic the MSC-based immunomodulatory and regenerative effects by delivering bioactive factors, such as proteins, nucleotides, lipids and so on.

EVs are nanoscale vesicles enwrapped by phospholipid bilayers and can be purified from various body fluids such as blood, urine, synovial fluid, and saliva[11]. It has been demonstrated that EVs play an essential role in cell-to-cell communication owing to their ability to encapsulate and deliver a variety of bioactive molecules, including proteins, lipids, mRNAs, microRNAs (miRNAs), and long noncoding RNAs, from parent cells to recipient cells[12]. The specific components of their contents vary with environmental conditions[13]. Almost all types of cells can generate and release EVs into extracellular space, which retain almost similar properties to their parental cells[14,15]. MSC-EVs in rheumatic diseases have drawn increasing attention in the last decade.

Currently, as there is no cure for RA and OA, searching for novel and effective treatment to attenuate pain and stop further damage has become a goal of the treatment of rheumatic diseases. Existing studies have demonstrated the significant advantages and great potential of MSCs and their EVs in immunomodulation and tissue damage repair. Targeting MSCs and MSC-derived EVs may be a more promising treatment for rheumatic diseases. This review summarizes recent advances in the functional roles and mechanisms of MSCs and EVs generated from MSCs in rheumatic disease, with a special focus on their potential therapeutic effects, providing rationalities for further research of MSCs and MSC-derived EVs in this field.

MATERIALS AND METHODS

Search strategy

The keywords of "mesenchymal stem cell, extracellular vesicle, autoimmunity, inflammation, rheumatoid arthritis, osteoarthritis, and rheumatic disease" were used alone or in combination to retrieve articles related to "immunomodulation" and "tissue regeneration and repair" in PubMed. Papers published in English language from January 1999 to February 2020 and available in full text were under consideration. Preliminary screening of papers concerning analysis of "immunomodulatory function" or "regenerative function" by scrutinizing the titles and abstracts of the literature, excluded the papers not related to the subject of the article. Some other related studies were obtained by manually retrieving the reference lists of papers that meet the selection criteria, and these studies were screened to meet the final selection and exclusion criteria.

Study eligibility criteria

The selection criteria were: (1) The subjects of research cover MSCs or EVs from MSCs with regard to mechanisms of immune regulation or tissue regeneration and repair; (2) The literature deals with relevant research and clinical application of MSCs or EVs from MSCs in the treatment of RA, OA, or other related diseases; (3) Articles recently published or published in authoritative and professional journals in the same field; and (4) High-quality articles with reliable arguments.

The following search records were excluded: (1) The content of research is repetitive and obsolete; (2) Literature unrelated to the treatment of MSCs or MSC-derived EVs

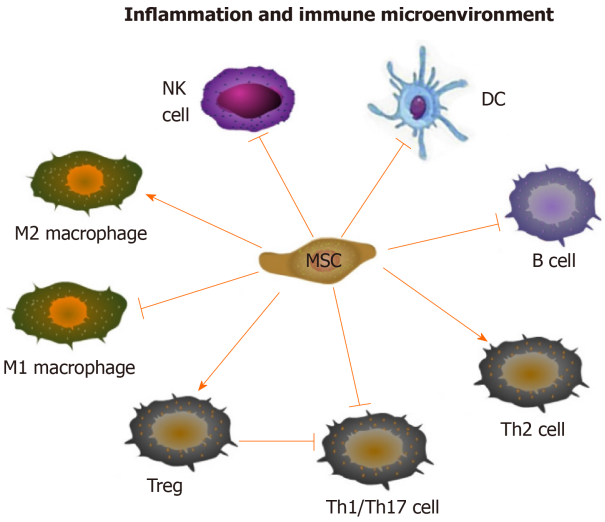

Figure 1 Immunomodulatory effects of mesenchymal stem cells. The inflammatory microenvironment stimulates mesenchymal stem cells (MSCs), leading to the acquisition or boost of their immunosuppressive property, then activated MSCs promote the conversion of pro-inflammatory macrophages (M1) into an anti-inflammatory phenotype (M2) and activation of Tregs as well as T helper 2 (Th2) cells, while inhibit the functions of Th1 cells, Th17 cells, B cells, dendritic cells, and natural killer cells. Actually, MSCs can also inhibit the amplification and differentiation of T cells indirectly *via* regulatory T cells. NK cell: Natural killer cell; DC: Dendritic cell; Th cell: T helper cell; Treg: Regulatory T cell.

for RA or OA; (3) Full text not available or those published in non-English language; and (4) Review, meta-analysis, and protocols.

Quality assessment

According to the inclusion criteria, two authors first scrutinized the titles and abstracts of the literature selected using the relevant keywords for preliminary screening to assess the effectiveness and applicability of the included literature. And to exclude articles that are inconsistent with the subjects of the study or that are repetitive, all authors read through the full text according to the exclusion criteria. Finally, 86 papers were selected for review and analysis.

Statistical analysis

This study is a systematic review of the literature, which did not involve any available statistical methods.

RESULTS

A total of 86 articles were included in the analysis after completing all the retrieval and review work (Figure 2). And a few articles were obtained by manually retrieving the reference lists of papers that comply with the selection criteria, and these studies were screened to meet the final selection and exclusion criteria. Figure 2 shows the process of literature retrieval. The great potential demonstrated in the literature of MSCs and MSC-derived EVs in modulating immune inflammation and promoting tissue regeneration supports their use in rheumatic disease.

At present, there is increasingly literature about the potential therapeutic value of MSCs in RA or OA. The application of MSCs in RA has primarily concentrated on their immunomodulation, and regenerative potential of MSCs has been intensively studied in experimental models of OA. A single intraperitoneal administration of MSCs could prevent further damage of articular bone and cartilage in a collagen-induced arthritis (CIA) mouse model representing human rheumatoid arthritis, proving that the joint protective effect is caused by the immunomodulation mediated by MSCs[16]. The beneficial effect of MSCs on RA is being gradually identified, from RA-like inflammatory models to refractory RA patients. Single intravenous injection of bone marrow-derived MSCs (BM-MSCs) to nine refractory RA patients without any other rheumatic diseases acquired a significant improvement of clinical symptoms[17]. The exciting results of MSC regenerative potential have been obtained in preclinical models as well as in patients with OA or damage of joint surface. The suppression of synovial activation, ligament related enthesophyte formation, and cartilage damage

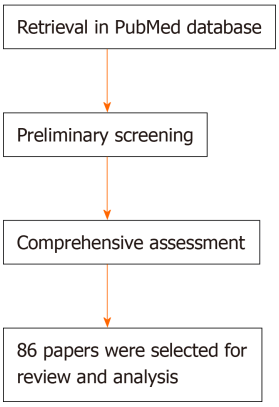

Figure 2 Flowchart for literature retrieval.

can be observed after intra-articular infusion of adipose tissue-derived MSCs (ADSCs) to mouse models with OA[18]. In addition, the similar effect of MSCs for cartilage regeneration also appeared in larger OA models such as the donkey and goat[19,20].

EVs isolated from MSCs, directly or loaded with therapeutics, such as specific miRNA[21], have also become the hotspot of recent research. Although MSC-derived EVs are not as commonly used in RA or OA as MSCs, it is clear that this cell-free therapy may become an alternative to MSC-based cell therapy. For example, MSC-derived EVs (exosomes and microparticles) efficiently ameliorated the inflammatory symptoms of CIA models *via* exerting an immunosuppressive effect on T-cell and B-cell[22]. In another study, MSC-derived exosomes were applied to rat models with osteochondral defects by intra-articular administration[23]. The results showed that the defects of rats in the experimental group recovered and finally proved the feasibility of MSCs in promoting cartilage repair. At present, research on MSC-derived EVs in RA and OA is far from enough, but a small part of the current research has aroused exciting interest.

DISCUSSION

MSCs and rheumatic diseases

MSCs are pluripotent progenitor cells that possess all the commonalities of stem cells, namely, self-renewal and multi-directional differentiation[24]. Over the last decades, MSCs are well known not only for their regenerative activity but also for their strong immunosuppressive property. MSCs can differentiate into three cell lineages of mesodermal organ *in vitro*, namely, osteoblasts, adipocytes, and chondrocytes[25]. Two important prerequisites for the application of MSCs in experimental research and clinical application are as follows: MSCs can be easily amplified *in vitro*; they can be present in a plenty of tissues including bone marrow[26], adipose tissue[27], Wharton's jelly[28], umbilical cord (UC-MSC)[29], umbilical cord blood[30,31], synovial membrane (SMSC)[32,33] and others, and among them bone marrow and adipose tissue are two commonly used tissues for therapeutic utilization[34]. Combing the above factors, MSC becomes the preferred seed cell for tissue engineering study.

A growing body of evidence has demonstrated that progressive immune inflammation contributes significantly in rheumatic diseases pathogenesis[35,36]. The chronic inflammation within the joint contributes to irreversible joint destruction. And the balance between joint destruction and tissue reconstruction as well as tissue repair determines the outcome of arthritis. It has been well known that MSCs can mediate a wide spectrum of immunoregulatory and tissue damage repairing activities, which support their use as a novel treatment option for rheumatologic disorders[37,38]. During the last few years, accumulating studies have been carried out to confirm the therapeutic value of MSCs for different rheumatic diseases, such as RA[39], OA[40], systemic lupus erythematosus[41], and ankylosing spondylitis[38]. Clarifying the mechanism of MSCs is crucial for identifying novel MSC-based strategies for these diseases.

Immunomodulatory effect of MSCs: MSCs have immunoregulatory bioactivity. The main immunological characteristics of MSCs are low immunogenicity and high

immunosuppressive ability. The precise molecular mechanisms of the immunomodulation effects of MSCs have not been fully elucidated. However, currently available data have suggested that MSCs play an immunosuppressive role mainly through intercellular contact and the secretion of soluble factors encapsulated by EVs[42]. Numerous MSC-EVs derived soluble factors can participate in the immunomodulatory process, such as nitric oxide (NO), prostaglandin E2 (PGE2), indoleamine 2-3-dioxygenase (IDO), IL-10, transforming growth factor (TGF)-β and so on[8] (Figure 1). They may come directly from MSC, or be produced by the paracrine of immune cells, including T cells, B cells, DCs, and NK cells. Accumulating studies have disclosed that MSCs can regulate immune and inflammatory response, including inhibition of proliferation and differentiation of T helper (Th)1, Th17 cells and B cells, induction of activation of Tregs, suppression of maturation of DCs, promotion of the polarization of macrophages to M2, and inhibition of the functions of NK cells[8] (Figure 1).

T cells: T cells are of critical importance in adaptive immunity, whose dysregulation contributes to the pathogenesis of rheumatic diseases. MSCs can prevent pathogenic T cell expansion and induce Tregs activation. Inhibiting the proliferation of Th1, Th17, and granulocyte-macrophage colony-stimulating factor-expressing $CD4^+$ T cells is the most significant effect of MSCs on T cells. Apart from depressing Th1/Th17 subtypes, MSCs also induce Th2, an anti-inflammatory subtype[43]. Human adipose tissue-derived MSCs have been shown to decrease the level of granulocyte-macrophage colony-stimulating factor-expressing $CD4^+$ T cells in peripheral blood and the spleen while increase the level of Tregs in CIA mice model[44], which suggests the immunosuppressive role of MSCs in suppressing $CD4^+$ T cells in RA pathogenesis[45]. The study by Ma *et al*[46] has demonstrated that human umbilical cord MSCs can reduce Th17 cell percentage *via* downregulating RORγt, and upregulate Foxp3 to augment Treg percentage in the spleen in RA[46]. Rashedi *et al*[47] have reported that MSCs can either increase the level of Treg cells by directly interacting with Tregs through the Notch signaling pathway or indirectly induce $CD4^+$ lymphocytes to differentiate into Treg cells[47]. In addition, bone marrow-derived MSCs can also inhibit the production of inflammatory cytokines by T cells in RA[48]. The significant anti-inflammatory role of MSCs on T cells is mainly dependent on hindering the nuclear factor-κB signaling pathway[49], which has been well recognized as the pivotal downstream signaling pathway involved in rheumatic disease pathogenesis[50]. Accordingly, the interaction between MSCs and T cells is involved in RA pathogenesis, providing novel strategies for the immunological treatment of rheumatic diseases.

B cells: MSCs can inhibit the multiplication and differentiation of B lymphocytes, even the production of immunoglobulins[51]. Che *et al*[52] has found that the suppressive effect of MSCs on B cell multiplication and differentiation is attributed to the downregulation of Blimp-1 and upregulation of PAX-5 in B cells[52]. Besides, it has also been well documented that MSCs exert effects on B cells by regulating interactions between programmed death 1 (PD-1) and its ligands PD-L1 and PD-L2[53]. MSCs can indirectly inhibit the effect of B cells through T cells[54]. Follicular helper T cells are also involved in the immunosuppressive process of MSCs by delivering proliferative signals to B cells in the secondary lymphoid tissues[55], which strongly supports that the suppressive activities of MSCs on B-cell also depend on the interaction between MSCs and T cells. Taken together, MSCs are involved in autoimmune disorders by influencing B cell proliferation, differentiation, and function.

Macrophages, DCs, and NK cells

MSCs play a role as immune suppressive cells in rheumatic diseases. MSCs can reprogram the functions of the macrophage by inducing the switch of activated macrophage from pro-inflammatory phenotype (M1) to an anti-inflammatory phenotype (M2)[4,56] *via* inhibiting nuclear factor-κb/p65 and activating signal transducer and activator of transcription 3 signaling pathways[57]. DCs, the main antigen presenting cells that initiate T cell immune response, have been widely recognized in regulating inflammation and autoimmunity. It has been confirmed that the inhibitory impacts of MSCs on lipopolysaccharide-elicited DC activation and maturation can be mediated by PD-L1 as well as NO, PGE2, and adenosine in canine immune-mediated disease models[58]. The blocking effect of MSCs on DC differentiation and maturation ultimately leads to inhibition of the T cell response[59]. Mediators of IDO and PGE2 generated by MSCs can restrain the extension and cytotoxicity of NK cells[60,61]. Nevertheless, little is known about the immuno-modulatory effect of MSCs in rheumatic disease mediated by intercellular

communications with macrophages, DCs, and NK cells. Elucidating this issue is essential for identifying the immunological targets for the diagnosis and treatment of rheumatoid disease.

Soluble cytokines

MSCs exert immunomodulatory function not only relying on cell-cell contact, but by means of producing multi soluble factors such as NO, PGE2, IDO, TGF-β1, tumor necrosis factor-inducible gene-6, and human leukocyte antigen-G5[62-64]. NO and PGE2 are essential for the suppression of T-cell expansion[65,66]. Additionally, MSCs derived soluble factors PGE2 and TGF-β1 also participate in inducing $CD4^+CD25^+Foxp3^+$ Tregs, which are also involved in inducing the transition of M1 macrophages to an anti-inflammatory M2 phenotype[43]. The immunoregulatory effect of MSCs can be enhanced upon exposure to interferon-γ under the inflammatory micro-environment[67-69]. Pro-inflammatory or anti-inflammatory mediators in the microenvironment can affect the function of MSCs[70]. Pretreatment of ADSCs with pro-inflammatory RASF enhances their ability to trigger Tregs and inhibit activated macrophages[70]. Some pro-inflammatory factors can sometimes interfere with the immunosuppressive effect of MSCs. The immunomodulatory property of MSCs is highly plastic in inflammatory microenvironment[71], in which the inflammatory cytokines act as a crucial switch, such as iNOS[72,73]. MSCs possess a pro-inflammatory phenotype and elicit inflammatory response through activation of TLR4 following exposure to inflammatory cytokines[73-75]. As a result, MSCs act as a double-edged sword in regulating immune responses. Given such plasticity in the immuno-modulatory effects of MSCs, in-depth research is needed to determine the application of MSCs in treating rheumatic disease.

Regenerative property of MSCs in rheumatic diseases: In recent years, the value of MSCs in the application of regenerative medicine has been deeply studied (for review, see[76]). In arthritis, the balance between joint destruction and repair determines the outcome of arthritis. Failure to tissue repair leads to joint damage and disability. Currently, MSCs provide a new prospect for the healing of arthritis in RA and OA. The mechanisms supporting the application of MSCs in promoting joint repair may be as follows: First, MSCs secret a large number of trophic factors to promote angiogenesis, anti-fibrosis, anti-apoptosis and so on; Second, MSCs differentiate into chondrocytes or osteoblasts directly. In short, the differentiation potential and paracrine effect of MSCs make them suitable for the repair of joint defects[77,78]. MSCs can differentiate into osteocytes and osteoblasts in osteoblast regulating medium containing inflammatory stimulants[79]. MSCs are capable of inhibiting osteoclast formation by enhancing the expression of osteoprotegerin[80], suggesting the critical role of MSCs in tissue regeneration.

Available data have revealed that intra-articular administration of MSCs can control synovial inflammation, reduce osteophyte formation, inhibit cartilage degeneration, and stimulate chondrocyte proliferation[18,81]. The important role of MSCs in OA cartilage regeneration has been well established in cartilage cells *in vitro*[82-84]. Besides, the regenerative potency of MSCs has been intensively studied in experimental models of OA and RA. Murphy *et al*[20] have first found that the administration of BM-MSCs exerts a regenerative effect in a caprine model with complete medial meniscus resection and anterior cruciate ligament resection[20]. The articular cartilage defect can be ameliorated by intra-articular infusion of MSC hyaluronic acid suspension in miniature pigs with condylar cartilage damage[85]. Similar use of MSCs has been investigated in other animal models of OA[19,86]. A clinical trial has recently reported that intra-articular administration of autologous ADSCs into the OA knee can improve the functional status, relieve pain, and reduce cartilage defects without side effects[87]. A two-year follow-up study conducted by Jo *et al*[88] has also demonstrated the safety and efficacy of intra-articular infusion of autologous ADSCs into the OA knee[88]. Accordingly, all these findings strongly support the regenerative efficacy of MSCs for promoting cartilage regeneration and protecting cartilage from degradation to impede the progression of arthritis. MSCs can be identified as a novel therapeutic strategy for those rheumatic disease patients particularly with arthritis and bone damages.

Several factors affecting the therapeutic effect of MSCs: The profound value of MSCs has aroused increasingly interests in immunomodulation and regenerative medicine, let alone in rheumatic disease. However, enormous challenges yet remain ahead of clinical application of MSC-based cell therapy due to their vulnerability. The action of MSCs, for instance, will differ according to MSC tissue origin, administration route, and others.

One of the most important reasons that MSCs can be extensively studied and applied is that MSCs can be purified from various tissues. But the most suitable cell source with the best therapeutic effect is still under study, due to the significant variation of MSCs from different sources in many aspects, including differentiation potential, immunomodulatory ability and so forth. BM-MSCs demonstrate a superior osteogenic and chondrogenic capacity, compared with ADSCs[89]. Another study reported that SMSCs exhibit a greater capacity for chondrogenesis *in vitro* over other four kinds of MSCs, in which BM-MSCs, ADSCs, and periosteal MSCs were included[90]. However, *in vivo*, the capacity in osteogenesis of SMSCs is inferior to that of periosteum-derived MSCs[91]. Furthermore, the influence of MSC tissue origin on immunomodulatory ability was demonstrated by Melief *et al*[92] - better immunosuppressive effect of ADSCs on T cells and monocytes than BM-MSCs was discovered in their study. No matter the variability between MSCs from different tissue sources, the similar immunosuppressive or beneficial effect to arthritis have been described[93-95].

The injection route of MSCs varies according to the pathological characteristics of different diseases. Generally speaking, diseases such as RA, which tend to involve multiple joints and are characterized by progressive inflammation caused by immune dysfunction, can be administered systematically (Figure 3). While diseases with limited lesions, such as OA, tend to be given locally. In contrast, part of research failed to demonstrate the improvement of arthritis by MSC-based treatment *via* systemic route[96,97].

The contradictory results show that the therapeutic potential of MSCs is disturbed by many factors other than tissue origin and administration route, which reveals the great challenge of current research, and more efforts are needed before MSCs can be put into clinical practice.

MSC-derived EVs in rheumatic diseases

EVs are well known for their great potential as a carrier for bioactive substances or biomarkers of diseases. According to their size and mode of biogenesis, EVs can be divided into three main categories: Exosomes, microparticles, and apoptotic bodies[98] (Table 1). Exosomes (30-120 nm in diameter) originate from intraluminal vesicles inside of multivesicular bodies, which fuse with the plasmolemma and release exosomes *via* exocytosis[99,100]. They are packed with tetraspanins (CD9, CD63, and CD81) and heat-shock proteins such as Hsp60, Hsp70, and Hsp90[10,101,102]. They also frequently express clathrin, alix, and tumor susceptibility gene 101[10,101,102]. The size of microparticles, known as microvesicles, ranges between 100 and 1000 nm. They are produced *via* budding directly from the plasma membrane of parent cells, which then are shed from the cell surface[10]. There are no specific surface molecular markers for microparticles, but they express the surface markers of parent cells like exosomes[103]. Apoptotic bodies (1000-5000 nm in diameter) are released by fragmenting apoptotic cells[104]. The well-established methods for isolating and purifying EVs include precipitation, differential ultracentrifugation, density gradient ultracentrifugation, ultrafiltration, size exclusion chromatography, and immunoaffinity[105]. EVs can encapsulate and deliver a variety of bioactive molecules, including proteins, lipids, and noncoding RNAs (ncRNAs)[12] from parent cells to recipient cells and participate in intercellular communications. Notably, miRNAs encapsulated by exosomes are a class of 20-22 nt small ncRNAs[106], which regulate targeted mRNAs at the post-transcriptional level *via* binding to 3′-untranslated region of the genes[106]. Previously, our team has demonstrated the specific miRNA expression profile in RA patients and shown that exosomal miR-6089 regulates inflammatory reaction in RA by targeting TLR4[107], which suggests the potential role of exosomal miRNAs as diagnostic biomarkers and treatment targets for RA. Nonetheless, the role of ncRNAs in MSC-derived EVs is unclear yet.

Recently, it has been supported that the effects of MSCs mediated by paracrine mechanisms are partly achieved through secretion of numerous EVs[108], although MSCs can also act directly (Figure 4). Growing evidence has revealed that EVs derived from MSCs also have immunomodulatory effects, and capacity of regeneration and repair of damaged tissues[80,109]. Vonk *et al*[109] have reported that EVs from BM-MSC can inhibit inflammation, promote regeneration, and repair cartilage damage *via* decreasing COX-2 and other pro-inflammatory factors when co-cultured with OA chondrocytes[109]. It has been recognized that EVs have the characteristics of selective assembly, targeted delivery, and stable preservation[110]. MSC-derived EVs have a significant effect in mediating immunomodulation and tissue repair. MSC-EVs not only recapitulate the therapeutic functions of MSCs[111], but also have many advantages that MSCs do not have. EVs are more stable in nature and stronger in transmission ability, compared with MSCs[112].

Table 1 Classification and characteristics of extracellular vesicles

	Exosomes	Microparticles	Apoptotic bodies
Size (diameter)	30-120 nm	100-1000 nm	1000-5000 nm
Mode of biogenesis	Originate from multivesicular bodies and released *via* exocytosis	Formed directly by the cell membrane outwards in the form of buds	Released from fragmented apoptotic cells
Content	Proteins, lipids, mRNAs, and miRNAs	Proteins, lipids, mRNAs, and miRNAs	DNA fragments
Molecular markers	Tetraspanins (CD9, CD63, and CD81), heat-shock proteins (Hsp60, Hsp70, Hsp90), alix, clathrin, tumor susceptibility gene 101	CD40, cholesterol, sphingomyelin, phosphatidylserine, ceramide	Annexin V, phosphatidylserine
Ref.	Keshtkar *et al*[115], Kanada *et al*[139]	Biancone *et al*[140], György *et al*[141]	Maumus *et al*[10], Kalra *et al*[104]

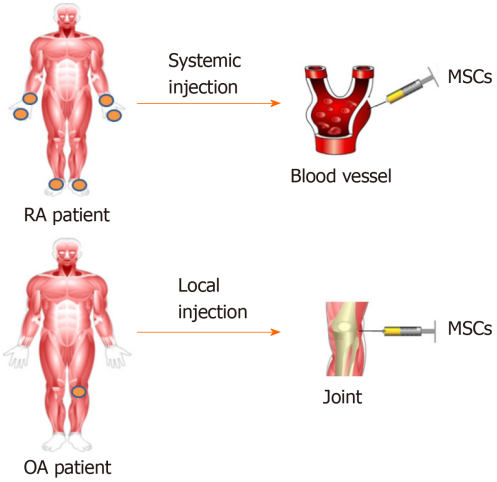

Figure 3 Administration routes of mesenchymal stem cells in rheumatoid arthritis and osteoarthritis. RA: Rheumatoid arthritis; OA: Osteoarthritis; MSCs: Mesenchymal stem cells.

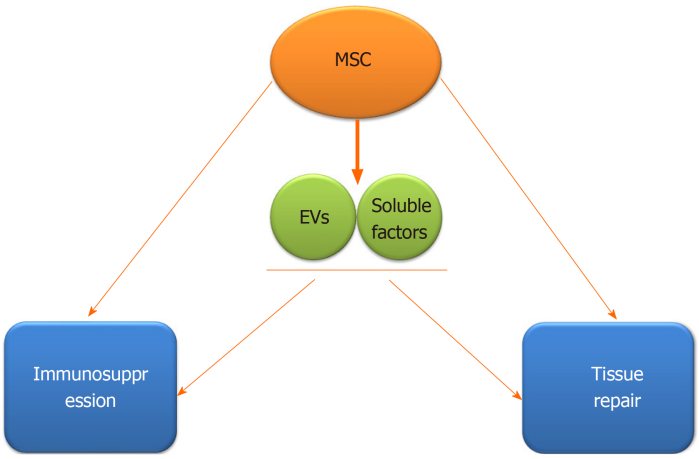

Figure 4 Schematic representation of whole action mechanisms of mesenchymal stem cells. MSC: Mesenchymal stem cell; EVs: Extracellular vesicles.

MSC-EVs encapsulate diverse lipids, proteins, miRNAs, and mRNAs that originate from MSCs and are secreted into the extracellular microenvironment. Accumulating studies have implicated that MSC-derived EVs exert effects *via* transporting molecules with biological activity[113]. Those EVs can interact with the recipient cells in a variety of ways, including fusing with the plasmolemma of recipient cells, interacting with target cell surface receptors, and internalizing by endocytosis, and subsequently deliver their

contents to receptor cells, therefore modifying inflammatory and immune responses[114,115].

Immunoregulatory property of MSC-EVs: Recently, the role of MSCs in immune regulation has been demonstrated by mounting studies[116,117], however, the application of MSCs in the clinic remains limited due to their instability. During the past few years, EVs derived from MSCs have attracted increasing attention. Accumulating studies have implicated that MSC-EVs also possess similar immunomodulatory property as MSCs[118,119]. MSC-EVs can also exert immunosuppressive effects on T cells[118], B cells[120], macrophages[121], DCs[122], and NK cells[123].

MSC-derived EVs are documented to restrain the multiplication of activated T cells and promote the production of tolerant Tregs[124]. Similarly, MSC-EVs can inhibit the activation and development of T cells by decreasing interferon-γ generated by $CD4^+$ T cells[125,126]. Exosomes from MSCs can also boost the production of $CD4^+CD25^+Foxp3^+$ Tregs[124]. Besides, MSC-derived exosomes have been found to inhibit inflammation by promoting the levels of anti-inflammatory cytokines IL-10 and TGF-β1 in PBMCs and inducing the activation of Tregs[127]. Reduced production of immunoglobulin and inhibited B cell proliferation and differentiation can be induced by MSC-EVs in B cells[128]. The immunosuppressive role of MSC-EVs in macrophages is also well established. EVs derived from MSCs can be effectively internalized by macrophages, which also suppress the pro-inflammatory phenotype (M1) macrophage activation but promote the anti-inflammatory phenotype (M2) macrophage activation[129]. However, the study by Monguio-Tortajada *et al*[130] has reported that EVs released by UC-MSCs do not affect the polarization of mononuclear macrophages[130]. The immunomodulatory effect of MSCs on peripheral blood leukocytes is significant[131,132], whereas no significant influence was observed in those leukocytes co-cultured with MSC-derived exosomes[133]. The difference in immunomodulatory mechanism between EVs and the receipt cells may be related to their tissue origins[118]. In summary, these findings provide the evidence for the immunoregulatory effect of MSC-EVs. Nevertheless, more studies are warranted for a clear understanding of the roles and mechanisms of MSC-EVs in immune regulations.

Regenerative effect of MSC-EVs: The regenerative action of MSCs-EVs has been well documented in a previous published study[111]. The critical role of MSC-EVs in tissue repairing is demonstrated in a femur fracture model of $CD9^{-/-}$ mice[134]. The study by Zhang *et al*[23] has first demonstrated that exosomes from human embryonic MSC confer a protecting effect on cartilage repair[23]. Exosomes released by both SMSCs (SMSC-Exos) and induced multipotent stem cell-derived MSCs (iMSC-Exos) can attenuate OA score in a mouse OA model, but a greater therapeutic effect of iMSC-Exos on OA than SMSC-Exos has also been demonstrated[126]. Similar to the effects of MSCs on inflammatory arthritis, MSC-EVs also can help to relieve the pain and joint damage in OA and RA *via* the protection against cartilage degradation.

Some well-established miRNAs delivered by exosomes have also been demonstrated in rheumatic diseases. MiR-320c-overexpressing hBMSC-Exos can induce cartilage regeneration in OA by promoting the proliferation of chondrocytes and decreasing MMP13 expression[135]. A similar effect of miR-140-5p-overexpressing SMSC-Exos has also been documented, which protect against OA[136]. Accordingly, MSC exosome-encapsulated miRNAs play a protective role in OA, which implicates a potential therapy for OA by targeting miRNAs delivered by MSC exosomes. However, more investigations are needed to clarify the underlying mechanisms of EVs in tissue damage, repair, and regeneration.

Although both MSCs and MSC-EVs have immunomodulatory and regenerative functions, the safety and efficiency of MSC-based cellular therapy should be seriously considered. Currently available data have demonstrated that the therapeutic activity of MSC-EVs may be superior to that of MSCs in terms of safety and versatility[115,137,138]. MSC-EVs offer a promising cell-free restorative approach for regenerative medicine and immunomodulation, which may be a better option for patients with OA and RA, and even other rheumatic diseases. Additionally, EVs can act as drug carriers by encapsulating and delivering small molecules and particular nucleic acids to targeted cells to acquire the desired therapeutic effect in rheumatic diseases. Chen and colleagues have elucidated that miRNA-150-5p delivered by MSC-exosomes plays a therapeutic role in RA through modulating MMP14 and VEGF[21]. Taken together[139-141], MSC-EVs-based therapeutic approach is promising for the treatment of rheumatic diseases because they offer the possibility to develop cell-free therapy.

At present, MSCs play important immunosuppressive and tissue regenerating roles through immune regulation, secretion of trophic factors, and multi-directional

differentiation, which has attracted much attention in the field of rheumatism. At the same time, as a product of MSCs, EVs have a similar function to MSCs, and may have more advantages than MSCs in biomanufacturing, storing, and other aspects, which makes it get more and more attention. MSC-EVs may represent a more promising therapeutic strategy in immune regulation and tissue repair and regeneration. In summary, MSCs and MSCs derived EVs can be novel therapeutic strategies in rheumatic diseases.

However, the current research is only the tip of the iceberg, from the point of view of clearly understanding the complete mechanisms of MSCs and EVs. At present, there are still many uncertainties in the precise roles and mechanisms of MSC-derived EVs in rheumatic diseases. Some current studies have shown that whether MSCs and EVs can play a full role in the treatment of diseases is affected by many factors. Obviously, in order to better understand their mechanisms of action, a large number of *in vivo* and *in vitro* studies need to be carried out in terms of tissue source, administration route, window of injection, injection dose and so on. Before application of MSCs and MSC-derived EVs into the treatment of rheumatic diseases, a large number of preclinical studies and clinical studies are required to thoroughly assess their safety and efficiency.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) have been widely investigated in rheumatic disease due to their immunomodulatory and regenerative properties. Recently, mounting studies have implicated the therapeutic potency of MSCs mostly due to the bioactive factors they produce. Extracellular vesicles (EVs) derived from MSCs have been identified as a prospective cell-free therapy due to low immunogenicity. Rheumatic disease, primarily including rheumatoid arthritis (RA) and osteoarthritis (OA), is a group of diseases in which immune dysregulation and chronic progressive inflammation lead to irreversible joint damage. Targeting MSCs and MSC-derived EVs may be a more effective and promising therapeutic strategy for rheumatic diseases.

Research motivation

MSCs and MSC-derived EVs have attracted increasing attention in rheumatic diseases due to their great potency in immunosuppression and tissue repair. Currently, it is of great significance to evaluate the therapeutic value by searching and summarizing the relevant literature.

Research objectives

To evaluate the potential therapeutic effectiveness of MSCs and EVs generated from MSCs in rheumatic diseases.

Research methods

One electronic database (PubMed) was searched for the relative literature using the corresponding search terms alone or in combination. Papers published in English language from January 1999 to February 2020 were in consideration. Preliminary screening of papers concerning analysis of "immunomodulatory function" or "regenerative function" by scrutinizing the titles and abstracts of the literature, excluded the papers not related to the subject of the article. Some other related studies were obtained by manually retrieving the reference lists of papers that comply with the selection criteria, and these studies were screened to meet the final selection and exclusion criteria.

Research results

Eighty-six papers were ultimately selected for analysis. After analysis of the literature, it was proved that both MSCs and EVs generated from MSCs exert great potential in multiple rheumatic diseases, such as RA and OA, in repair and regeneration of tissues, inhibition of inflammatory response, and regulation of body immunity *via* promoting chondrogenesis, modulating innate and adaptive immune cells, and regulating the secretion of inflammatory factors. But EVs from MSCs exhibit much more advantages over MSCs, which may represent another promising cell-free restorative strategy. Targeting MSCs and MSC-derived EVs may be a more efficient treatment for patients with rheumatic diseases.

Research conclusions

MSCs and MSC-derived EVs have demonstrated powerful regenerative potency, as well as their regulatory function for the innate and adaptive immune system. This study offers new ideas and possibilities for MSCs and EVs from MSCs to rheumatism treatment due to their enormous potential described above. However, more in-depth exploration is needed before their clinical application.

Research perspectives

The great potency of MSCs and MSC-derived EVs has been demonstrated, and they can be developed as a more effective and promising therapeutic strategy for rheumatic diseases. Before application of MSCs and MSC-derived EVs into the treatment of rheumatic diseases, a large number of preclinical studies and clinical studies are required.

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