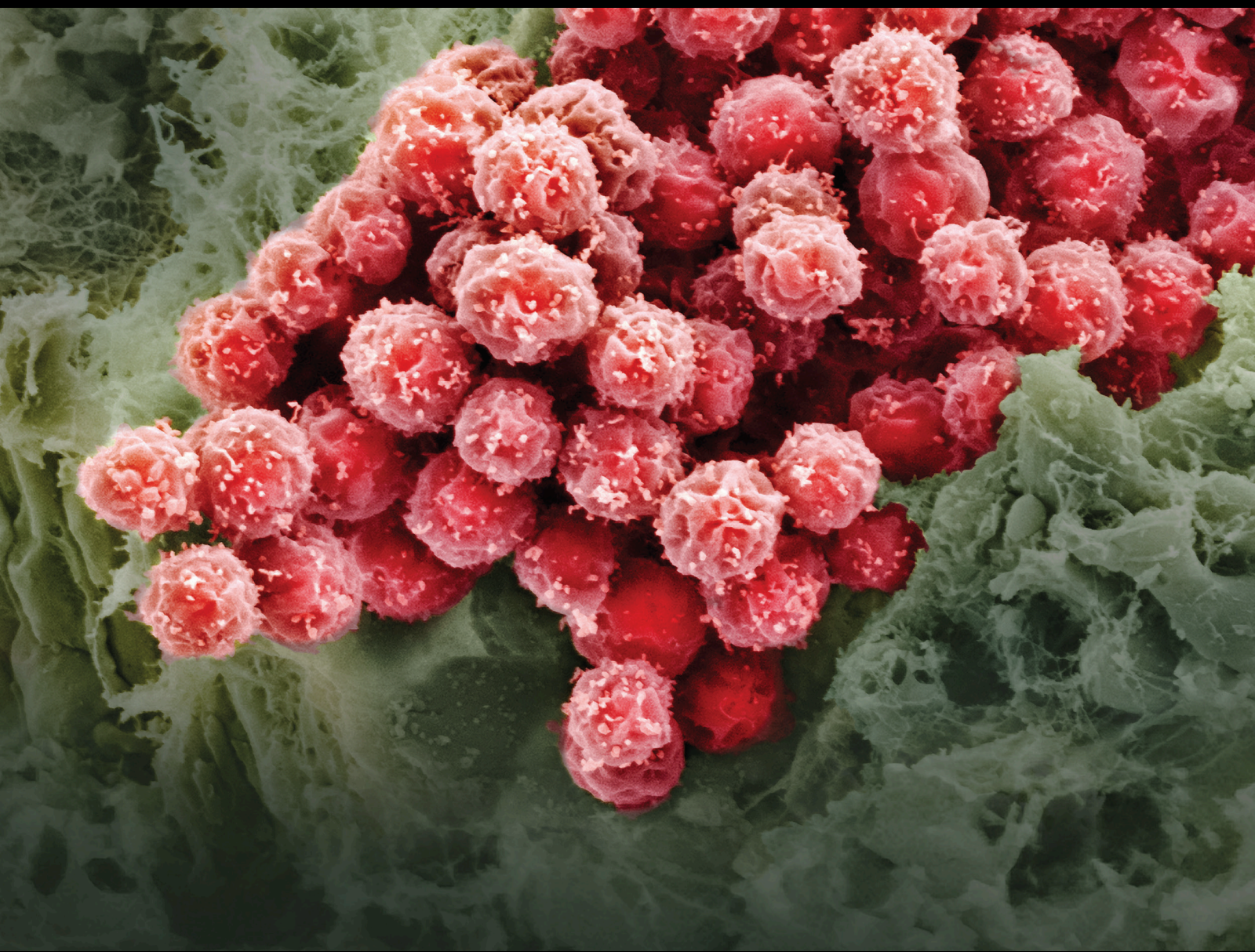
Stem Cells International

Improving the Therapeutic Ability of Mesenchymal Stem/Stromal Cells for the Treatment of Conditions Influenced by Immune Cells

Guest Editors: Lindolfo da Silva Meirelles, Marcela F. Bolontrade, Melissa Medeiros Markoski, Bruno Dallagiovanna, and Laura Alaniz



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Editorial

Improving the Therapeutic Ability of Mesenchymal Stem/Stromal Cells for the Treatment of Conditions Influenced by Immune Cells

Lindolfo da Silva Meirelles,[1] **Marcela F. Bolontrade**,[2] **Melissa Medeiros Markoski**,[3] **Bruno Dallagiovanna**,[4] **and Laura Alaniz**[5]

[1]*PPGBioSaúde, Lutheran University of Brazil, Canoas, Brazil*
[2]*IMTIB-CONICET-IUHI-HIBA, Buenos Aires, Argentina*
[3]*Federal University of Health Sciences of Porto Alegre, Porto Alegre, Brazil*
[4]*Carlos Chagas Institute–Fiocruz Paraná, Curitiba, Brazil*
[5]*CIT NOBA–CONICET-UNNOBA, Junín, Argentina*

Correspondence should be addressed to Lindolfo da Silva Meirelles; lindolfo.meirelles@ulbra.br

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Mesenchymal stem/stromal cells (MSCs) have been initially described decades ago as fibroblastic precursors that could be isolated from the bone marrow and establish cultures of fibroblastic cells. These fibroblastic cells were shown to support hematopoiesis in vitro, which is a characteristic of stromal cells, and, later, to give rise to mature mesenchymal cells such as bone, cartilage, and fat cells when cultured under appropriate conditions. The proposition that a mesenchymal stem cell exists in postnatal bone marrow and other tissues as blood vessel-associated cells provided further momentum to research on these cells, as well as divergences on how to call them. The impetus of using MSCs to replace cells lost in various types of conditions eventually decreased, as the therapeutic benefits provided by these cells were found to be mostly due to the secretion of paracrine signaling molecules, which can be carried by extracellular vesicles. In the meantime, MSCs were found to modulate the behavior of immune cells by means of secretion of molecules that could, in different scenarios, inhibit the activation of T cells that promote adaptive immune responses. Subsequently, the effects of MSCs on other cells of the immune system were also described. Today, a number of clinical trials using MSCs to treat conditions influenced by immune cells are under way. While preclinical data indicates that MSCs have important immunomodulatory properties, further studies are still in progress to increase the knowledge on the differences regarding the action of MSCs on immune cells according to their tissue of origin, on how MSCs exert their effects on the different types of immune cells, and on ways to improve the outcome of conditions influenced by immune cells when treated using MSCS.

This special issue was open to basic research manuscripts and reviews that approached ways to improve the therapeutic ability of MSCs for the treatment of conditions influenced by immune cells. Accordingly, two basic research papers on the interactions between MSCs and immune cells in skin wound models and three reviews on aspects of the relations between these cells were accepted. S. Xiao et al. showed that forced expression of interleukin-10, an anti-inflammatory cytokine, in human amnion-derived MSCs improves the healing of full-thickness skin wounds in mice by reducing inflammation and excessive extracellular matrix deposition while improving angiogenesis. He and his colleagues focused on the effects of exosomes, a type of extracellular vesicle, produced by MSCs isolated from human bone marrow on the polarization of macrophages in full-thickness skin wounds of mice. Consequently, X. He et al. found that microRNA-223 present in these exosomes contribute to a proregenerative M2 polarization in macrophages. B. S. Guerrouahen et al. reviewed mechanisms underlying communication of MSCs with

immune cells and discussed clinical applications of MSCs in diseases mediated by immune cells. F.V. Paladino et al. reviewed the immunomodulatory properties of Wharton's jelly-derived MSCs. Finally, Z. Fábián discussed the effects of hypoxia on the immunomodulatory properties of the bone marrow. This group of articles provides a contribution to better understand and, consequently, improve the therapeutic properties of MSCs for the treatment of conditions influenced by the immune system.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

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Lindolfo da Silva Meirelles
Marcela F. Bolontrade
Melissa Medeiros Markoski
Bruno Dallagiovanna
Laura Alaniz

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Review Article

The Effects of Hypoxia on the Immune-Modulatory Properties of Bone Marrow-Derived Mesenchymal Stromal Cells

Zsolt Fábián [1,2]

[1]*Department of Medicinal Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary*
[2]*School of Medicine, Faculty of Clinical and Biomedical Sciences, University of Central Lancashire, Preston, UK*

Correspondence should be addressed to Zsolt Fábián; zsolt.fabian.dr@gmail.com

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Guest Editor: Lindolfo da Silva Meirelles

The therapeutic repertoire for life-threatening inflammatory conditions like sepsis, graft-versus-host reactions, or colitis is very limited in current clinical practice and, together with chronic ones, like the osteoarthritis, presents growing economic burden in developed countries. This urges the development of more efficient therapeutic modalities like the mesenchymal stem cell-based approaches. Despite the encouraging *in vivo* data, however, clinical trials delivered ambiguous results. Since one of the typical features of inflamed tissues is decreased oxygenation, the success of cellular therapy in inflammatory pathologies seems to be affected by the impact of oxygen depletion on transplanted cells. Here, we examine our current knowledge on the effect of hypoxia on the physiology of bone marrow-derived mesenchymal stromal cells, one of the most popular tools of practical cellular therapy, in the context of their immune-modulatory capacity.

1. Introduction

Mesenchymal stromal cells (MSCs) are considered to be a promising tool for cellular therapy in various human pathologies. These include both chronic and acute inflammatory conditions like, for instance, osteoarthritis, rheumatoid arthritis, colitis, septic conditions, or graft-versus-host disease. Despite numerous studies indicating the efficacy of MSCs in inflammatory animal models, clinical trials reported controversial outcomes. Behind the diverse pathogenesis of the distinct inflammatory conditions, local hypoxia is considered to be a common pathogenic factor. Indeed, inflammation is often accompanied by metabolic hypoxia in various inflammatory diseases. Bone marrow-derived MSCs (BMSCs) naturally reside in a severely oxygen-depleted microenvironment that supports the concept of their use in the cellular therapy of inflammatory conditions [1, 2]. Since differential oxygen levels exert complex effects on cellular physiology, here, we review our current understanding on the interplay between the immune-modulatory effects and hypoxic response of BMSCs and formulate problems to be addressed in order to develop more efficient BMSC-based medical applications for inflammatory pathologies.

2. Bone Marrow-Derived Mesenchymal Stromal Cells

BMSCs, similar to mesenchymal stem cells isolated from other tissues, are multipotent cells that possess the plasticity to differentiate into various cell types of mesenchymal origin [3, 4]. It is noteworthy, however, that some studies on BMSC plasticity widened the range of tissues BMSCs which could be potentially differentiated further [5–9]. These data suggest the existence of trans-lineage plasticity in BMSC populations and raise the question if BMSCs, or at least a subset of these cells, are rather pluripotent. Independent of this classification/semantical uncertainty, their plasticity fueled the idea that they have great medical potential in pathologies affecting tissues with poor regenerative capacity like the cartilage, myocardium, or tendons [10]. In support of this concept, intra-articular administration of BMSCs to patients suffering from knee cartilage damage was reported

beneficial based on clinical scorings, though the fate of transplanted cells remained unevaluated [11]. Another study found that the use of hyaluronic acid augments the effects of transplanted BMSCs indicating that the importance of the surrounding microenvironment in the efficacy of the BMSC-based cellular therapy [12]. In contrast, however, no statistically significant improvement was reported in osteoarthritis patients after cellular therapy with BMSCs differentiated toward chondrogenic lineages prior transplantation raising the question if efficacy observed in trials was mediated by direct cartilage repair [13]. Indeed, tissue damage is often accompanied by inflammation so one can speculate that for successful tissue regeneration, transplanted cells have to, ideally, modulate the inflammatory milieu. Clinical reports on the efficient use of hBMSCs in high-risk pediatric acute leukemia patients to improve platelet and neutrophil recovery, apparently, support this hypothesis. Although data are not consistent among published clinical trials, BMSCs were considered to be responsible for the apparent attenuation of the graft-versus-host reactions, possibly, through their anti-inflammatory effects posttransplantation [14, 15]. An independent phase I/II study, however, reported that the majority of the patients either showed partial response or did not respond to BMSC-based cellular therapy at all [16].

Similarly, conflicting results have been published in relation to other inflammatory conditions as well. *In vivo* studies on the potential use of BMSCs in inflammatory conditions of the respiratory system showed promising results. In rodent smoke-induced lung damage models, rat BMSCs (rBMSCs), administered via the trachea, repressed the expression of proinflammatory cytokines tumour necrosis alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and the monocyte chemoattractant protein 1 (MCP-1) in the lung parenchyma. Parallel, induction of the vascular endothelial growth factor (VEGF), its type 1 receptor (VEGFR1), and the transforming growth factor beta (TGF-β) was reported in lung tissue homogenate suggesting an overall anti-inflammatory pulmonary effect of rBMSCs [17]. In a follow-up study, the same group reported repression of cyclooxygenase-2 (COX-2) and its downstream effector prostaglandin E2 (PGE2) production in alveolar macrophages as a possible mechanism behind the rBMSC-mediated anti-inflammatory pulmonary effects [18]. Independent studies in mouse lipopolysaccharide-induced pneumonia models also suggest that BMSCs may mediate the anti-inflammatory effects through the modulation of macrophage functions [19–21]. Despite the promising results in animal models, however, a multicenter phase II study in over 60 chronic obstructive pulmonary disease (COPD) patients did not find significant effects of the use of intravenous infusions of allogenic human BMSCs (hBMSCs) [22]. In accordance, only weak efficacy of the intravenous transplantation of hBMSCs was observed in a recent phase I trial with patients suffering from acute respiratory distress syndrome (ARDS) raising the question of both the mechanisms underlying the controversial responses and optimized protocols for improved therapeutic efficacy [23].

Despite the likely diverse extracellular milieu present in distinct inflammatory conditions, one could speculate that the determining factor of the BMSC-based cellular therapy outcome is the differential oxygen levels cells are exposed to before, during, and after transplantation. Indeed, $CD4^+$ T cells, for instance, adapt successfully to hypoxic conditions and this adaptation is accompanied by elevated secretion of a cohort of proinflammatory cytokines including IL-1β, IL-6, IL-8, IL-10, and MCP-1 [24]. BMSCs are also naturally resistant to a severely oxygen-depleted environment, but cells that are transplanted, for instance, into joints have to exert their immune-modulatory functions in a fundamentally differentially oxygenated milieu compared to those trapped in the lungs after intravenous administration [25]. Thus, understanding the adaptation of BMSCs to various oxygen levels might be one of the keys for establishing better off-shelf BMSC products and more efficient BMSC-based therapeutic protocols for inflammatory diseases.

3. Hypoxia

Although hypoxia is typically associated with pathophysiologic states, it is, actually, present in physiologic conditions as well. Indeed, oxygen depletion occurs from the very first stages of embryogenesis and remains present during the whole morphogenesis. Local hypoxia not only is responsible for the proliferation of placental epithelial stem cells, the cytotrophoblasts, but also serves as an orientation signal for their invasion into the uterus, a critical factor of placental development [26]. Hypoxic tissues are present in the growing embryo elsewhere as well, and their common presence in various experimental model species including rodents and birds suggests that the phenomenon is a general property of the vertebrate embryogenesis [27]. Although its distribution shows a temporospatial variation, hypoxic regions remain detectable throughout the whole morphogenesis. In the 14.5E mouse embryo, for instance, extensive oxygen-depleted regions are present in the midbrain, pituitary gland, spine cord, vertebrae, and sternum as well as in tissues of the tongue, heart, lungs, and intestine [28]. *In vivo* data also showed that artificial modification of oxygen levels upon embryonic development leads to severe placental malformations or abnormal morphogenesis suggesting that the embryonic hypoxia cannot be exclusively considered a passive outcome of the massive expansion of embryonic tissues but rather a tightly regulated organogenetic signal [29, 30]. This also underlines the importance of the hypoxic milieu in the physiology of pluripotent cellular species responsible for tissue organogenesis. Extremely low oxygen tensions are also present in tissues under physiologic condition during the postembryonic life. This "physiologic" hypoxia is present even in well-vascularized organs like the heart, kidneys, or brain (Table 1.). Moreover, recent findings on the central role of the microbiome-mediated oxygen-depletion of the intestinal epithelium in the maintenance of the intestinal barrier function suggest that the physiologic role of hypoxia in adult tissues might be more critical than it has been, previously, anticipated [31].

3.1. The Molecular Machinery of Hypoxic Adaptation. Independent of the nature of hypoxia, metazoan cells need to adapt to the oxygen-depleted milieu to ensure the balance of their oxygen-dependent metabolic homeostasis and

Table 1: Oxygen concentrations of various tissues.

Tissue/organ	O_2 (%)	Reference
(i) Lung parenchyma (ii) Circulation (iii) Well-irrigated parenchymal organs	4-14	[96–104]
(i) Brain tissue	0.5-7	[105–108]
(i) Retina (ii) Corpus vitreum	1.0-5	[109, 110]
(i) Bone marrow	0-4	[1, 111]

survival. Adjustment of cellular metabolism in hypoxia is, primarily, orchestrated by helix-loop-helix type transcription factors termed hypoxia-inducible factors (HIF) [32] (Figure 1). The heterodimer HIFs, besides the shared beta one, consist of distinct alpha subunits that are steadily degraded by the 26S proteasome system in oxygenated cells [33]. This "normoxic" degradation is facilitated by the hydroxylation of conserved proline residues of the α polypeptides mediated by the prolyl-4-hydroxylase-1, prolyl-4-hydroxylase-2, and prolyl-4-hydroxylase-3 (PHD1, PHD2, and PHD3) [34]. Hydroxylation renders α subunits bound to the E3 ubiquitin ligase component von Hippel-Lindau (pVHL) protein leading to their proteasomal breakdown and absence of functional heterodimers in "normoxic" cells [33]. Under hypoxia, in contrast, PHDs become inactive, HIF-α subunits stabilize and dimerize with their β counterparts and transactivate adaptive target genes. These include not only genes of the glucose and lipid metabolism but also the ones encoding for regulators of proliferation, survival, DNA repair, cytoskeletal components, extracellular matrix-related proteins, cyto-, and chemokines [35]. Since BMSCs express both HIF-1 and HIF-2α and the HIF-orchestrated cellular hypoxic response is fully functional in these cells, one can speculate that differential activation of the underlying molecular system consequently affects the putative immunomodulatory nature of these cells too [36].

4. The Hypoxic Response of BMSCs

4.1. Metabolic Adaptation. One of the critical aspects of the HIF-governed hypoxic adaptation is the metabolic switch from the oxidative phosphorylation to less oxygen-dependent metabolism. Since in BMSCs both the aerobic glycolysis and oxidative phosphorylation are active and the HIF system is also intact and functional, one can speculate if the HIF-orchestrated metabolic switch remains active in the *ex vivo* expanded BMSC cultures [37]. *In vitro*, it, apparently, does since hBMSCs exposed to 2% oxygen show elevated glucose consumption compared to cells cultured under atmospheric oxygen conditions [38]. Parallel, the incorporation of glucose-derived carbons into citrate, which reflects the rate of the glycolysis-driven TCA cycle, is significantly reduced. Despite this reduction, however, citrate carbons are still mainly derived via pyruvate dehydrogenase indicating that basal activity of the TCA cycle remains intact even under oxygen-depleted conditions [39]. Interestingly, although this metabolic switch under hypoxia should also be reflected in lactate production, there are contradicting results in relation to lactate production of hypoxic BMSC cultures. While some studies observed decreased extracellular lactate levels in the culture media of oxygen-deprived BMSCs, others reported elevated lactate production under hypoxic conditions [37, 38, 40]. Recent systemic analyses of the hypoxic BMSC metabolome detailed the picture further showing that elevated extracellular lactate levels are accompanied by unchanged intracellular lactate levels suggesting the existence of a high-capacity lactate export system in BMSCs [39]. Since lactate export seems to become saturated upon *in vitro* osteogenic differentiation, one may wonder if a differential proportion of undifferentiated species in the BMSC cultures examined is accounted for the reported conflicting results in lactate production.

In glutamate metabolism, which serves as carbon and nitrogen supplies alike, hypoxic BMSCs display different kinetics as well. Under hypoxia, they show an increase in TCA cycle-driven metabolism of glutamate and this, in conjunction with the elevated glucose consumption, may be related to the activated malate-aspartate shuttle observed [39]. Data suggest that this metabolic profile allows hypoxic BMSCs to maximize their ATP production at reduced glycolytic carbon supply of the TCA cycle. In addition, increased glutamate metabolism in oxygen-deprived BMSCs is accompanied by reduced production of ammonia, the by-product of glutamate metabolism, suggesting that glutamate conversion is, primarily, mediated by transaminases instead of the ammonia-producing glutamate dehydrogenase in hypoxic BMSCs [38, 39]. Since the transaminase pathway of glutamate metabolism facilitates generation of nonessential amino acids, one can speculate that the increased glutamine consumption of hypoxic BMSCs mainly serves their translational machinery [41]. This is in accordance with the findings that oxygen-depleted BMSCs secrete a number of soluble factors with potential impact on the inflamed microenvironment and the hypoxic glutamine metabolism may serve the reprogrammed translation of hypoxic BMSCs. It is also noteworthy that normoxic cultures are reported to produce ammonia at concentrations that are believed to be inhibitory *in vitro* so one can speculate if the hypoxia-adapted glutamine metabolism with reduced ammonia production is reflected in the proliferative capacity of *ex vivo* expanded BMSC cultures [42].

4.2. Proliferation of Hypoxic BMSCs. Indeed, BMSC cultures expanded at oxygen levels lower than 3% are reported to show better proliferative capacity and consistently higher cumulative population doublings compared to cells kept under atmospheric oxygen conditions [36, 38, 43]. This may be critical for BMSC-based therapeutic applications since these modalities require *ex vivo* expansion of cells to be transplanted due to the low frequency of BMSCs in source marrow isolates [4]. Analyses of proliferation kinetics revealed that hypoxic cells enter the cell cycle faster and start *in vitro* cell division earlier than that of the normoxic ones [38]. Although details of the underlying mechanisms including the role of reduced production of ammonia are still not fully understood, a number of parallel events, which may

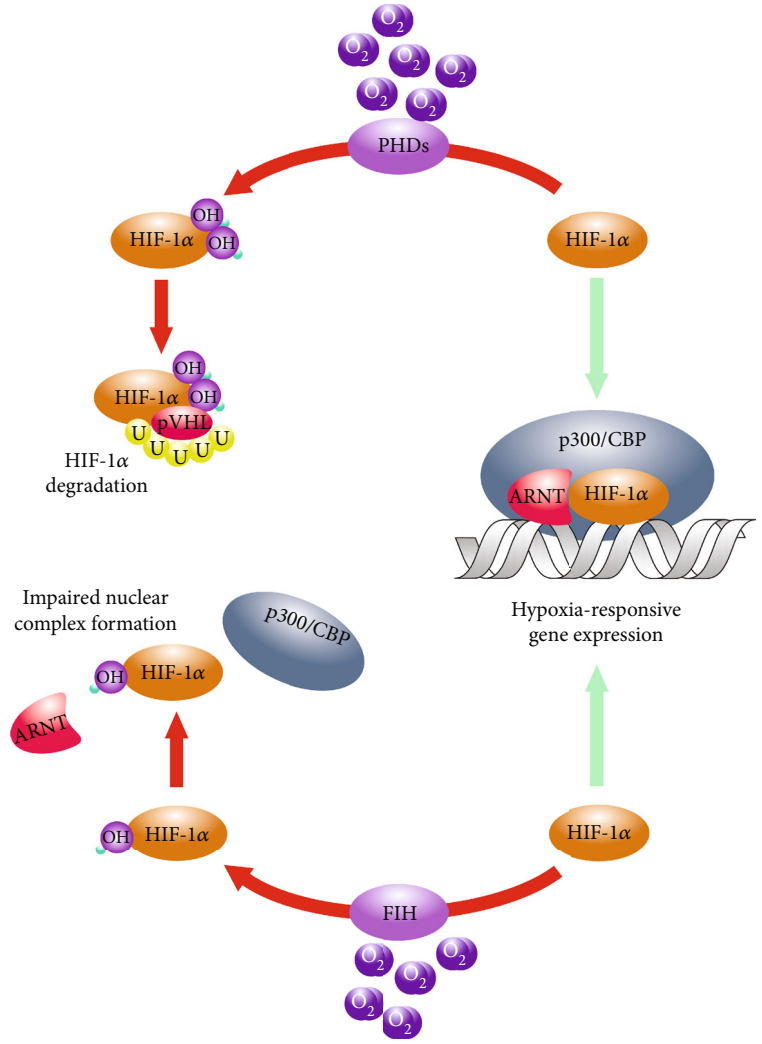

Figure 1: Hydroxylation-mediated regulation of the HIF-α subunits. The primary posttranslational regulation of the HIF-α polypeptides is mediated by the prolyl-4-hydroxlase-1, prolyl-4-hydroxlase-2, and prolyl-4-hydroxlase-3 (PHDs) that catalyze the hydroxylation of conserved proline residues. This leads to the ubiquitylation and subsequent proteasomal degradation of the HIF-α subunits in the presence of oxygen. A complimentary hydroxylation catalyzed by the asparagine hydroxylase termed factor inhibiting HIF (FIH) that prevents the association of HIFs with their transcriptional coactivator p300.

potentially orchestrate the hypoxia-driven upregulation of BMSC proliferation, have already been reported.

One of these mechanisms is mediated by the APELIN-AKT/PKB axis in hypoxic BMSCs (Figure 2) [43]. APELIN is the endogenous ligand for the orphan G protein-coupled receptor APJ, and the APELIN-encoding *APLN* gene is induced in a HIF-1α-dependent manner in hypoxic BMSCs [44, 45]. *In vitro* studies using rodent BMSCs revealed that APELIN-mediated activation of APJ leads to the inactivating phosphorylation of glycogen synthase kinase 3 beta (GSK3β) via the AKT/PKB in a phosphoinositide 3-kinase- (PI3K-) dependent manner [43, 46]. One of the known targets of GSK3β is cyclin D1, the regulatory component of the cyclin D1/cyclin-dependent kinase 4 (CycD1/CDK4) complex that governs the G_1/S phase transition in the cell cycle [47]. The GSK3β-mediated phosphorylation of cyclin D1 results in nuclear export and the cytoplasmic degradation of the latter one leading to inactivation of the CycD1/CDK4 complex. Thus, experimental data suggest that, in hypoxic BMSCs, the HIF-induced *APELIN* triggers the AKT/PKB axis that results in the inactivation of GSK3β and, consequently, upregulation of the CycD1/CDK4 complex and the G_1/S phase transition [46]. Since in, cancer cells, AKT/PKB-mediated inactivating phosphorylation of GSK3β contributes to the cytoplasmic stabilization of HIF-1α as well, one can speculate if an APELIN-AKT/PKB-HIF-1α axis forms a feed-forward regulatory loop in hypoxic bone marrow-derived mesenchymal stromal cells [48]. Moreover, since the translational regulator mammalian target of rapamycin (mTOR) is also a known effector of AKT/PKB in established cellular models, it would be interesting to see how the hypoxia-upregulated AKT/PKB contributes to the altered ammonia production via, for instance, the mammalian target of rapamycin (mTOR) pathway in hypoxic BMSCs.

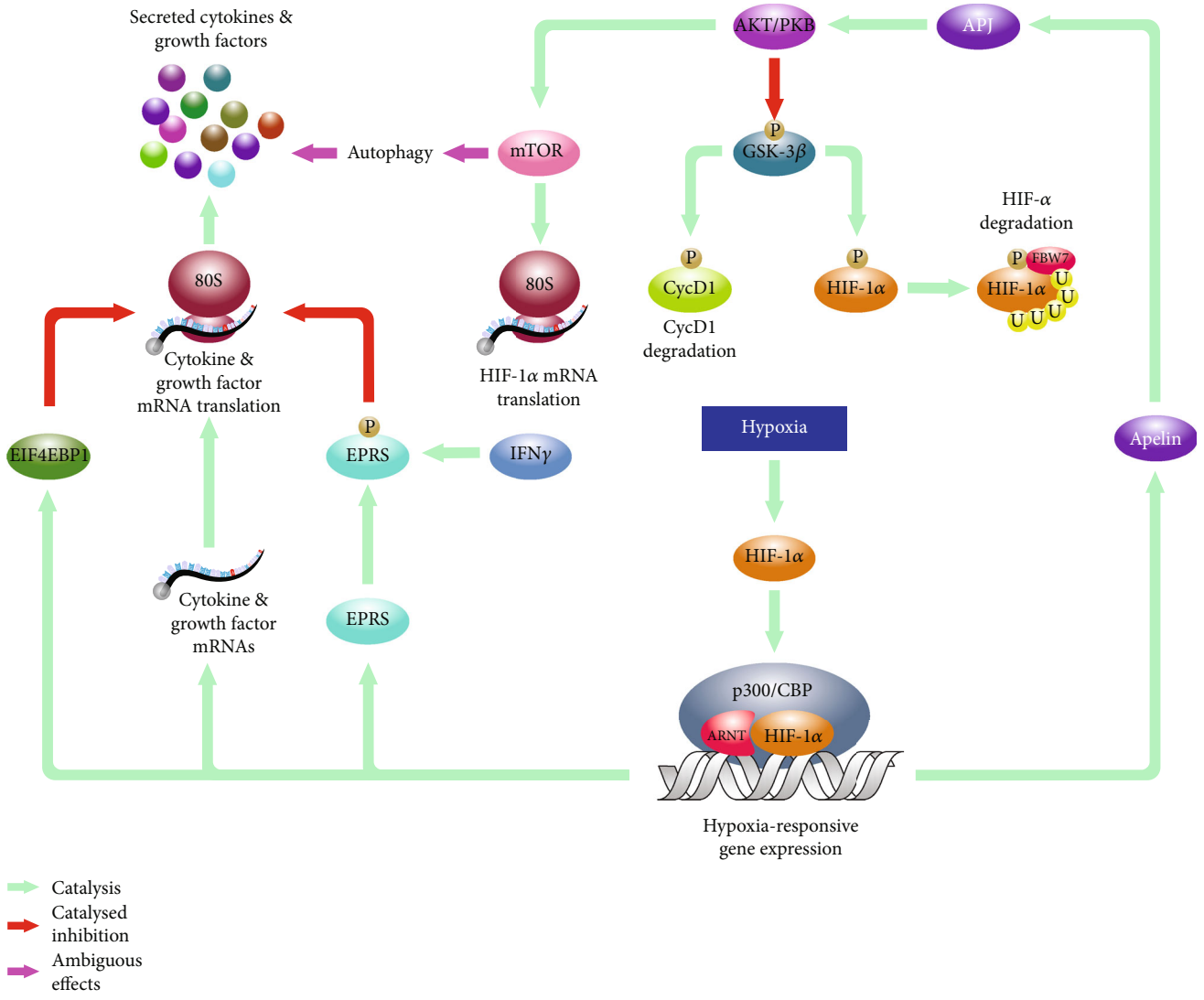

Figure 2: Molecular mechanisms involved in the proliferative and cytokine response of hypoxic BMSCs. Hypoxia-stabilized HIFs induce genes like *APLN* that, in return, activate the AKT/PKB pathway. This leads to inactivating phosphorylation of GSK3β releasing cyclin D1 from GSK3β-mediated inhibition. Data also suggest that the activation of the AKT/PKB results in the regulation of mTOR that affects both autophagic and translational activities of BMSCs. Besides mTOR, hypoxia also induces genes like *EPRS* and *IEF4EBP1* that also contribute to the hypoxia-specific translational pattern, likely, to define composition of secreted immune-modulatory factors of BMSCs.

4.3. Cytokine Production of Hypoxic BMSCs. Hypoxia-stabilized HIFs target hundreds of genes mostly inducing their expressions. This leads to complex modification of the gene expression pattern of BMSCs as it has been shown using oxygen-depleted rBMSCs [49]. Target genes include those encoding for proteins with known or predicted secretory functions that may exert immune-modulatory effects [43, 50]. One of the potential mediators of these effects is the robustly hypoxia-induced macrophage migration inhibitory factor (*MIF*) that, although traditionally has been considered to be a proinflammatory cytokine, can function as a mediator of the monocyte/macrophage arrest as well by acting as a noncognate ligand for the chemokine receptors CXCR2 and CXCR4 [51]. Another candidate target is *PTGES* that encodes for the prostaglandin E synthetase, suggesting elevated PGE2 synthesis in hypoxic BMSCs. PGE2 has been reported to support monocyte differentiation into type 2 macrophages (Mϕ2) that are known activators of regulatory T lymphocytes (T_{reg}) [52]. Since this raises the fact that BMSCs apply their immune-modulatory effects, at least in part, via the PGE2-Mϕ2-T_{reg} axis, it would be interesting to see if differential expression of the transforming growth factor beta (TGFβ), which also promotes T_{reg} formation, exists in hypoxic BMSCs and if so, it contributes to the immune-modulatory properties of hypoxic BMSCs [53]. Apparently, this concept is underpinned by the findings that hypoxic mBMSCs trigger both proliferation and viability of the Mϕ2 fraction via a cell-to-cell contact mechanism that is, at least in part, mediated by M-CSF and ICAM-1 [54].

Whether hypoxia mediates similar alterations in the gene expression profile of human BMSCs and, if so, how these differentially regulated genes contribute to the observed immune-modulatory effects of BMSCs in inflammatory conditions need further investigations. However, not only *bona*

fide secretory proteins may have a role in the immune-modulatory effects observed in relation to BMSCs. Indeed, hypoxia upregulates *EPRS* that encodes the glutamyl-prolyl-tRNA synthetase. Although it is primarily known as a cytoplasmic enzyme that catalyzes aminoacylation of glutamate and proline tRNA species, it also suppresses translation of diverse inflammatory mRNAs by binding their 3′-UTRs upon interferon-gamma-mediated phosphorylation [55]. Moreover, since proteolytic fragmentation of the tyrosyl-tRNA synthetase generates polypeptides that affect neutrophil chemotaxis by binding the CXCR1 chemokine receptor, one can speculate if hypoxic upregulation of the glutamyl-prolyl-tRNA synthetase in BMSCs has similar immune-modulatory functions [56].

The complex effects of hypoxia on the translational regulation of BMSCs are further indicated by the hypoxic induction of the eukaryotic initiation factor 4E-binding protein 1 (EIF4EBP1), a suppressor of 5′-CAP-dependent translation observed in rBMSCs [50, 57]. In established cell lines, oxygen depletion activates the AKT/PKB pathway that leads, among others, to the activation phosphorylation of the mTOR. As it has been discussed above, in mouse BMSCs (mBMSCs), the proximal section of the putative AKT/PKB-mTOR-EIF4EBP1 axis is activated by the hypoxia-inducible *APLN* [45]. Since mTOR is a known regulator of EIF4EBP1, one may wonder if the hypoxia-responsive, translational pattern-regulating AKT/PKB-mTOR-EIF4EBP1 axis exists in human BMSCs [50]. The finding that the hypoxia-mediated secretion of soluble factors like VEGF, FGF2, IGF-1, and HGF is sensitive to PI3K inhibitor 3-methyladenine (3-MA) in mBMSCs, apparently, supports the concept that a hypoxia-responsive AKT/PKB-mTOR-EIF4EBP1 pathway participates in the translation of cytokines/growth factors [58].

Interestingly, recently, it was also reported that siRNA-mediated knockdown of ATG7 attenuates the increased secretion of growth factors that suggests an interplay between the upregulation of growth factor secretion and ATG7-governed functions like, for instance, vacuole transport or autophagy [58]. Since autophagy, which is traditionally concerned as an mTOR-governed process, contributes to cell survival, the role of ATG7 in the cytokine secretion suggests a potential link between the immune-modulatory effects and viability of hypoxic BMSCs as well.

4.4. Hypoxia Affects Viability of BMSCs. One of the most profound effects of hypoxic exposure on BMSCs is shifted proliferation that raises the question if hypoxic exposure leads to premature senescence and, thus, exhausted immune-modulatory capacity of BMSC cultures. Apparently, some experimental data support this concern as far as the relative telomere length of hypoxic BMSCs was found shorter than that of the cells kept under atmospheric oxygen conditions [38]. In accordance, some studies reported an increased rate of apoptosis in BMSC cultures kept under hypoxia [59–61]. Still, it is widely believed that viability is preserved in *bona fide* hypoxic BMSC cultures as illustrated by the increased colony-forming unit values observed in hypoxic BMSC cultures [38, 62]. In accordance, hypoxia-stabilized HIF-1a has been shown to mediate the survival of rBMSCs in the presence of exosomes derived from oxidative stress neuronal cells [63]. One possible explanation for this controversy is that, in studies which reported elevated cell death, hypoxia was combined with serum deprivation so the observed apoptotic response may be accounted for the lack of vital nutrients rather than to low-oxygen levels. This notion is underpinned by the elevated glucose and glutamine consumption of hypoxic BMSCs discussed above. In terms of shortened telomeres reported in hypoxic BMSCs, data indicate that compensating prosurvival mechanisms may sustain viability of hypoxic cells. Indeed, both expression of LC-3, BECLIN-1, and ATG5, hallmarks of autophagy, and conversion of LC3B-I to LC3B-II, a marker of autophagosome formation, were reported in mBMSCs exposed to hypoxia [64]. The finding that induction of autophagy markers is sensitive to U0126, the selective inhibitor of the MAP kinases MEK1 and MEK2, indicates that hypoxia-triggered activation of autophagy is, at least in part, mediated by the MAPK pathway in mBMSCs [64]. The putative role of the MAPK pathway in the hypoxic response seems to be evolutionarily conserved as hypoxic activation of the ERK pathway has been shown in human BMSCs as well [36].

The potential importance of hypoxia-triggered autophagy may be illustrated by the observation that a short-term hypoxic exposure of mBMSCs protects cells from subsequent hypoxia/serum deprivation injury [58]. The protective effect of hypoxic preconditioning, in accordance with human models, is accompanied by increased levels of LC3 and BECLIN-1 further supporting the evolutionarily conserved aspect of the hypoxia-mediated upregulation of autophagy markers in BMSCs. Seemingly, induced autophagy makes mBMSCs more resistant to environmental stress. Indeed, hypoxia preconditioned mBSMCs are reported to show better survival after transplantation to infarcted hearts or when exposed to H_2O_2 [58, 65]. In support of this concept, HIF-1α overexpression, which may mimic hypoxic preconditioning, has also been shown to protect rBMSCs from oxygen-glucose deprivation-induced damage and this effect was correlating with the expression of autophagy markers [66]. Experimental data on non-preconditioned ischemic mBMSCs indicate that autophagy cannot rescue ischemic cells from apoptosis without mTOR activity and suggest that hypoxic preconditioning mediates resistance by upregulation of mTOR, probably, via the HIF-1α-APLN-AKT/PKB axis in the advancement of ischemic exposure [59]. Interestingly, shRNA-mediated knockdown of *ATG7* increased viability of hypoxic human BMSCs suggesting that, at least in hypoxic mBMSCs, ATG7 is not necessary to the hypoxia-responsive autophagy-mediated prosurvival mechanisms [67]. This observation also suggests that the role of ATG7 in hypoxia-responsive secretion of growth factors is more closely related to the vesicular transport functions of ATG7 than to its role in autophagosome formation.

This is in accordance with findings that indicate the importance of vesicular transport in BMSC-mediated immune-modulatory functions. Indeed, BMSC-derived exosomes have been reported to affect proliferation of cocultured cells and stem cell-derived exosomes have also been found to exert immune-modulatory effects [68, 69]. These data also

question if live BMSCs are actually needed to reach the desired therapeutic effects in the cellular therapy of inflammatory conditions. Indeed, even ischemia-treated annexin V/propidium iodide-positive mBMSCs were shown to have immune-modulatory effects on cocultured macrophages [70]. The observed repression of inflammatory cytokines TNF-α, IFN-γ, IL-12, and IL-6 and induction of PGE2, VEGFA, angiopoietin 1 (Ang-1), keratinocyte growth factor (KGF), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor B chain homodimers (PDGF-BB), and erythropoietin (EPO) in cocultured macrophages indicate that even damaged BMSCs could reprogram the cytokine/growth factor profile of surrounding phagocytes. The general perception of controversies between the lasting immune-modulatory effects and the short half-life of transplanted BMSCs together with the absence of recipient BMSCs in heart and lung transplants or the observations that intravenously administered BMSCs are mostly trapped in the lungs posttransplantation is, apparently, in accordance with the idea that BMSCs can exert their immune-modulatory effects, at least in part, indirectly [71–76]. In accordance, coculture experiments with damaged BMSCs suggest that immune-modulatory effects are, at least in part, accounted for phagocytotic capacity saturated by the cellular debris of transplanted BMSCs [69]. Interestingly, despite the fact that it is widely accepted that *ex vivo* culturing influences the phenotype and surface antigen pattern of BMSC cultures without making them immunogenic and that exosome-mediated horizontal transfer of the anti-inflammatory BMSC phenotype is an exciting potential mechanism for mediating the anti-inflammatory effects, little is known on the effects of the *ex vivo* expansion of BMSCs on their interplay with resident phagocytes posttransplantation [77, 78]. Accordingly, it would also be exciting to see if various *ex vivo* oxygen levels have any impact on the anti-inflammatory properties of BMSCs via, for instance, expression of neoantigen.

5. Conclusions

The discovery of multipotent species in adult tissues paved the way for the clinically efficient regenerative medicine. The idea that transplanted stem cells repair damaged tissues via their plasticity, however, has, slowly, been shifted to the concept that multipotent cells exert their biological effects indirectly. Apparently, this notion makes them particularly useful to treat inflammatory conditions, where soluble factors play pivotal roles. Still, clinical trials delivered perplexing results calling further investigations for understanding the mechanism of action of stem cell's immune-modulatory effects as well as for conditions that improve the efficacy of stem cell-based therapeutic modalities in inflammatory pathologies.

Indeed, over the past decades, BMSC-based cellular therapies have drawn great attention in the clinical practice. Indeed, BMSCs have been tried in a number of human pathologies that exert immune dysfunction or imbalance of the regulation of immune response where our current therapeutic repertoire is very limited. Still, despite promising preclinical data, clinical trials failed to deliver breakthrough results. A good example is graft-versus-host disease (GVHD) where BMSCs were used in a number of, mostly phase I and II, clinical trials for the treatment of both acute and chronic forms of GVHD. Unfortunately, while the use of BMSC-based cellular therapy in acute GVHD was reported advantageous by a number of reports, clinical trials found the same approach rather ineffective in patients who suffered from chronic GVHD [16, 79–83]. Multiple sclerosis (MS), which affects the central nervous system by demyelination of the motor axons, is another autoimmune pathology where no effective treatment is currently available. Progressive MS patients treated with BMSCs, however, showed partial responses, some degree of remyelination in affected CNS areas, and improved T_{reg} lymphocyte titers suggesting that cellular therapy may have genuine therapeutic potential in MS following improvement of its efficacy [84, 85]. Similar conclusions can be drawn from clinical trials targeting patients suffering from steroid-refractory systemic lupus erythematous, a potentially fatal multisystem autoimmune disease. These trials showed that BMSC infusions maintained patients in remission up to 18 months with elevated T_{reg} lymphocyte numbers [86] but simple repetition of BMSC transplantation did not improve the efficacy of the therapy [87].

Inflammation is always accompanied by hypoxia raising the question if differential oxygen levels throughout the therapeutic processes influence the immune-modulatory capacity of naturally hypoxic BMSCs. Indeed, data indicate that, in hypoxia, BMSCs are biasing their metabolic homeostasis toward aerobic glycolysis. This, combined with the observed glutamine-mediated anaplerosis, not only enables faster ATP generation in the absence of full-blown oxidative phosphorylation but also provides a range of metabolic intermediates that can fuel *de novo* synthesis of essential biomolecules, critical prerequisites of cell survival, translation, and secretory functions [88].

Considering their phenotypic analogy, it is not surprising that hypoxic BMSC metabolism resembles the one observed in cancer cells that also often show an extremely high rate of glutamine consumption and dependency [89]. Today, it is also widely accepted that inflammation is tightly linked to tumour formation and recent advances in immunotherapy of neoplasms substantiate the notion that tumour cells exert immune-modulatory properties. In addition, the striking variation in the immune profiles of distinct tumours suggests that transformed cells apply multiple mechanisms to attenuate immune reactions [90]. Indeed, cancer cells are reported to be able to recruit anti-inflammatory cells like T_{reg} lymphocytes and myeloid-derived suppressor cells or secrete soluble immunosuppressive factors like TGFβ, IL-10, or PD-L1 [91]. Since BMSCs are expected to exert their immune-modulatory functions in pathologies with similarly diverse inflammatory backgrounds, it seems to be a fascinating question if any of the immune-modulatory mechanisms of cancer cells apply to BMSCs.

Indeed, loss of function mutations of TP53, for instance, attenuates cytotoxic T cell invasion of breast cancers [92]. In a number of cancers, the absence of physiologic TP53 functions activate the nuclear factor kappa B (NF-κB) pathway, that is, typically, accompanied by the paralysis of

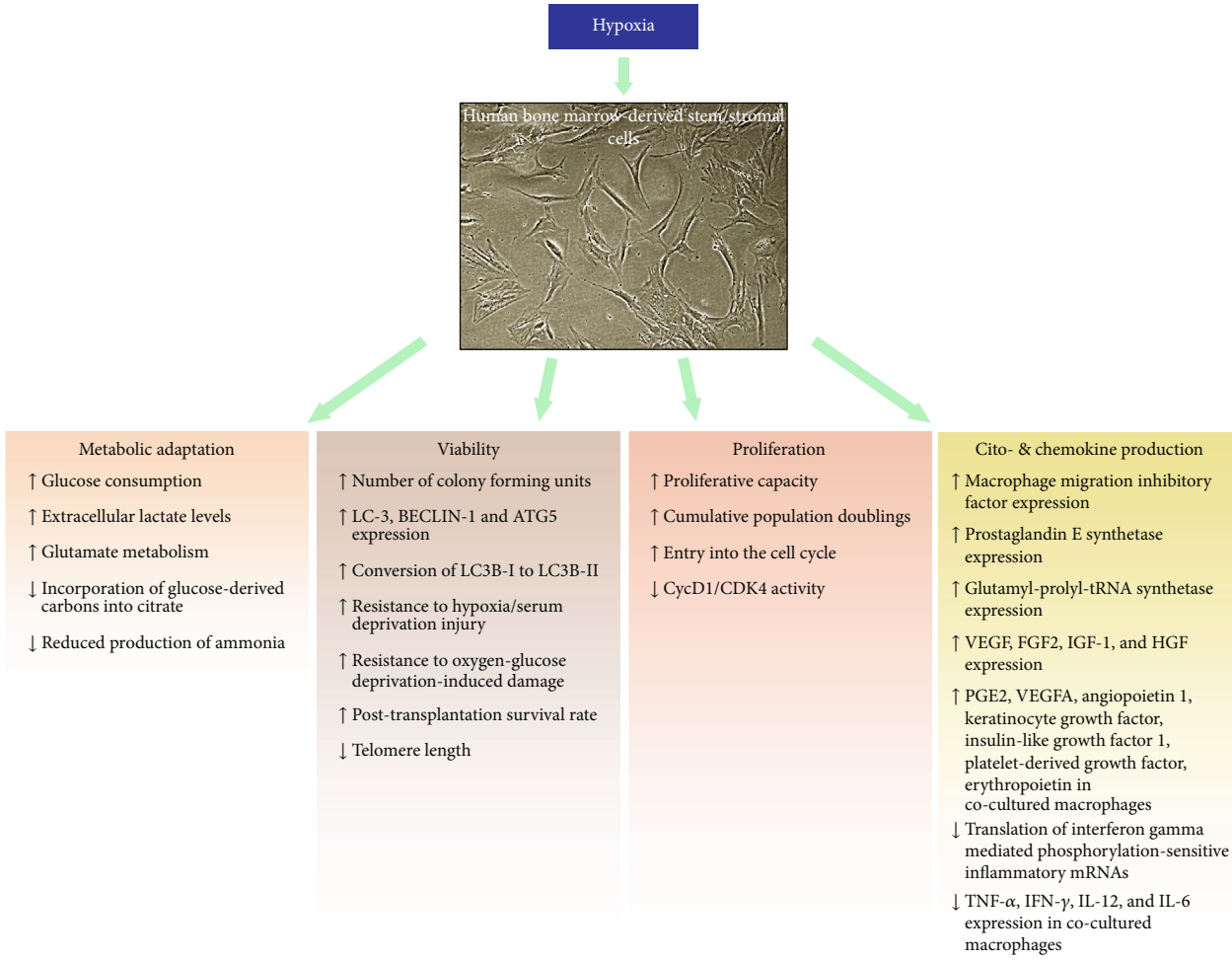

Figure 3: Summary of the key effects of hypoxia on BMSCs. The micrograph depicts human bone marrow-derived mesenchymal stem cells cultured under 2% oxygen in the absence of fibroblast growth factor-2 taken by the author using phase-contrast microscopy at 40x magnification.

immune cell influx of tumour mass [93]. Since some data suggest that TP53 may be repressed in hypoxic BMSCs as well, one may wonder if the NF-κB pathway, the critical mediator of inflammatory responses, is upregulated in hypoxic BMSCs modulating their cytokine/chemokine production [61]. Since the activity of the NF-κB pathway in hypoxic cells, apparently, depends on the cytokine profile of the extracellular milieu, one can hypothesize that the putative TP53-mediated, hypoxia-responsive activation of NF-κB contributes to the cytokine/chemokine production of hypoxic BMSCs. In return, this may downregulate the same in neighboring hypoxic immune cells modulating their cytokine/chemokine profile and, thus, reactivity [94].

Current data support the idea that hypoxic exposure of BMSCs pretransplantation may be one of the measures that improve their immune-modulatory effects posttransplantation (Figure 3). Data, however, also indicate that the prerequisite of an optimal hypoxic preconditioning protocol is the appropriate supply of nutrients like glucose and glutamine in order to fuel the hypoxia-reprogrammed translation of BMSCs with necessary metabolites. Nevertheless, the careful selection of supplements is underlined by the observation that ascorbic acid (AA) promotes BMSC proliferation [95]. Though the primary underlying mechanism is not clear due to its promiscuous metabolic roles, data indicate that exogenous AA mimics the effects of extracellular collagen fibers via increased collagen production, affects metabolism, and alters DNA methylation in BMSCs. Since AA, among others, acts as one of the cofactors of PHDs, one can speculate that AA might counteract the HIF-mediated mechanisms. Indeed, ascorbic acid, apparently, overrides the transcriptional activity of HIF triggered by deferoxamine (DFO), a routinely used hypoxia mimetic that, as an iron chelator, blocks the iron-dependent PHDs and therefore stabilizes HIFs. Since HIF transcriptional activity seems to be critical in the unfolding of the hypoxic BMSC phenotype, these observations illustrate that differential *ex vivo* culture conditions may provoke fundamentally different molecular mechanisms even in the presence of apparently equivalent macroscopic phenotypes.

These findings underline the importance of further optimization of the treatment regimens including manufacturing standards for future BMSC products. Experimental data not only indicate that activation of the molecular hypoxia-

adaptive machinery can significantly contribute to the efficacy of BMSCs in inflammatory pathologies but also underline the importance of further research on the optimal *ex vivo* conditions, including hypoxia, for establishing enhanced anti-inflammatory BMSCs. Indeed, careful selection of the oxygen levels during isolation, *ex vivo* culturing, and posttransplantation seems to be one of the key aspects we need to consider in order to improve the efficacy of the clinical use of BMSCs. Hopefully, future *in vivo* studies focusing on the role of oxygen in BMSC-based cellular therapies of inflammatory conditions will answer this question.

Conflicts of Interest

The author declares no conflict of interest.

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Research Article

MSC-Derived Exosome Promotes M2 Polarization and Enhances Cutaneous Wound Healing

Xiaoning He,[1,2] Zhiwei Dong,[3] Yina Cao,[4] Han Wang,[4] Shiyu Liu,[1,2] Li Liao,[1,2] Yan Jin,[1,2] Lin Yuan,[4] and Bei Li[1,2]

[1]*State Key Laboratory of Military Stomatology & National Clinical Research Center for Oral Diseases & Shaanxi International Joint Research Center for Oral Diseases, Center for Tissue Engineering, School of Stomatology, Fourth Military Medical University, Xi'an, Shaanxi 710032, China*
[2]*Xi'an Institute of Tissue Engineering and Regenerative Medicine, Xi'an, Shaanxi 710032, China*
[3]*State Key Laboratory of Military Stomatology & National Clinical Research Center for Oral Diseases & Shaanxi Engineering Research Center for Dental Materials and Advanced Manufacture, Department of Oral Implants, School of Stomatology, The Fourth Military Medical University, Xi'an, Shaanxi 710032, China*
[4]*Department of Stomatology, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 510140, China*

Correspondence should be addressed to Lin Yuan; 1549058757@qq.com and Bei Li; lbfmmu@163.com

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Mesenchymal stem cell transplantation (MSCT) promotes cutaneous wound healing. Numerous studies have shown that the therapeutic effects of MSCT appear to be mediated by paracrine signaling. However, the cell-cell interaction during MSCT between MSCs and macrophages in the region of cutaneous wound healing is still unknown. In this study, early depletion of macrophages delayed the wound repair with MSC injection, which suggested that MSC-mediated wound healing required macrophages. Moreover, we demonstrated that systemically infused bone marrow MSCs (BMMSCs) and jaw bone marrow MSCs (JMMSCs) could translocate to the wound site, promote macrophages toward M2 polarization, and enhance wound healing. *In vitro* coculture of MSCs with macrophages enhanced their M2 polarization. Mechanistically, we found that exosomes derived from MSCs induced macrophage polarization and depletion of exosomes of MSCs reduced the M2 phenotype of macrophages. Infusing MSCs without exosomes led to lower number of M2 macrophages at the wound site along with delayed wound repair. We further showed that the miR-223, derived from exosomes of MSCs, regulated macrophage polarization by targeting pknox1. These findings provided the evidence that MSCT elicits M2 polarization of macrophages and may accelerate wound healing by transferring exosome-derived microRNA.

1. Introduction

Mesenchymal stem cells (MSCs) are an enticing potential therapeutic agent for a variety of inflammatory reactions, including those that occur during wound healing. Mesenchymal stem cell transplantation (MSCT) is currently being used as a cellular therapy to promote cutaneous wound healing [1–3]. During cutaneous wound healing, most of the therapeutic benefits of MSCT appear to be derived from the release of paracrine factors, which stimulate differentiation and angiogenesis [1]. The cell-cell interaction also plays an important role in promoting wound healing during MSCT [3, 4]. However, the interaction of MSCs and other cells which functionally affect cutaneous wound healing remains to be elucidated.

Although widely recognized as the contributors of the early inflammatory response, monocytes and macrophages also contribute to angiogenesis, wound contraction, and tissue remodeling, which are required in the wound-healing process [5, 6]. In response to activation signals, macrophages are polarized toward an M1 phenotype (proinflammatory) or an M2 phenotype (anti-inflammatory). Accumulating evidence shows that M2 macrophages can express mediators that are essential in the resolution of inflammation and tissue

remodeling and, thus, promote wound healing [7, 8]. Several studies have demonstrated that MSCs can modify macrophages from the M1 to the M2 phenotype *in vitro* and *in vivo* [4, 9]. However, the underlying mechanism of the MSC-guided transition of macrophages from the M1 to the M2 phenotype during wound healing is still unknown.

Recently, MSCs have been found to secrete significant amounts of small vesicles (40-100 nm), known as exosomes following fusion of multivesicular endosomal membranes with the cell surface [10, 11]. Exosomes are emerging as a new mechanism for cell-to-cell communication and played an important role in wound repair [12, 13]. They carry a variety of proteins, mRNAs, and microRNAs, all of which may functionally modify recipient cells that interact with exosomes. We hypothesized that exosomes derived from bone marrow-derived mesenchymal stem cells (BMMSCs) mediate the polarization of the M2 macrophage during wound repair.

2. Materials and Methods

2.1. Animals and Ethical issues. Adult C57BL/6J mice (female, 6 to 8 weeks old) were obtained from the Laboratory Animal Research Center of the Fourth Military Medical University. Animals were maintained under good ventilation and a 12 h light/dark cycle and kept feeding and drinking *ad libitum* before being sacrificed. Mice were anesthetized with 1% pentobarbital sodium (200 mg/kg) via intraperitoneal administration and kept at an anesthetized state during surgery. Animals were euthanized by exsanguinations after receiving intravenous injections of MSCs or exosomes.

All animal procedures were performed according to the guidelines of the Animal Care Committee of Fourth Military Medical University (IRB-REV-2015005), and all experimental protocols were performed with the approval of the Fourth Military Medical University.

2.2. Cell Cultures. Human jaw bone marrow-derived mesenchymal stem cells (JMMSCs) and BMMSCs were isolated and identified as previously described [14]. Briefly, JMMSCs and BMMSCs were collected from bone marrow aspirates of the jaw bone and iliac crest, respectively. Bone marrow aspirates were collected, and the cells were plated into 6-well culture dishes (Costar®; Corning Inc., Corning, NY, USA) in an α-minimal essential medium (α-MEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co. Ltd., Zhejiang, China), 0.292 mg/mL L-glutamine (Invitrogen Life Technology, Carlsbad, CA, USA), 100 units/mL penicillin (Invitrogen), and 100 mg/mL streptomycin (Invitrogen) at 37°C under 5% CO_2. Cells were cultured about 2 weeks and the medium was changed after every three days. We used BMMSCs and JMMSCs at passages 2-5 (P2-P5) in this study. We further identified the capacity of proliferation of these MSCs by MTT assay (ATCC, Manassas, VA, USA). The MSC positive markers CD105, CD73, and CD90 or negative markers, CD14, CD19, HLA-DR, CD34, and CD45 (BD Biosciences, San Diego, CA, USA), were measured using flow cytometric analysis. The capacity for multipotent differentiation, including osteogenic and adipogenic differentiation, was detected by alizarin red staining and western blotting for Runx2, SP7 (Santa Cruz Biotechnology, Dallas, Texas, USA), COL-1, and ALP (Abcam, Cambridge, UK) and by Oil Red O staining and western blotting for PPAR-γ and LPL (Abcam, Cambridge, UK).

Human monocytes were isolated from the peripheral blood of normal human volunteers (blood donors from the Blood Transfusion Department of Xijing Hospital) using a Human Monocyte Isolation Kit II (Miltenyi Biotec, Teterow, Germany). In brief, peripheral blood mononuclear cells were collected by density gradient separation using a Lymphocyte Separation Medium (TBD Science Biotech Company, Tianjin, China). Red blood cells were lysed by incubating cells in a red blood cell lysis buffer (BioFlux, Beijing, China) for 3 min, and mononuclear cells were washed with PBS. Then, cell pellets were resuspended and incubated with anti-human CD14 antibody (eBiosciences, San Diego, CA, USA) for 10 min and biotin-labeled microbeads (Miltenyi Biotec, Teterow, Germany) for 15 min at 4°C degree. Purified $CD14^+$ monocytes were plated into 6-well cell culture plates at a concentration of $0.5\text{-}1 \times 10^6$ per well in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. Zhejiang, China).

2.3. Isolation and Characterization of Exosomes. To avoid contamination of serum exosomes, cells were cultured in a complete medium depleted of FBS-derived exosomes by ultracentrifugation at 100,000 g for 3 h at 4°C. Ten milliliters of culture supernatant was collected to isolate exosomes with ExoQuick-TC (ExoQuick; System Biosciences), according to manufacturers' protocol. Briefly, the supernatant was centrifuged at 3000 g for 15 min, mixed with 2 mL ExoQuick-TC exosome precipitation solution, and incubated for over 12 h at 4°C. Then, the mixture was centrifuged at 1500 g for 30 min. The samples were then loaded onto a carbon-coated electron microscopy grid and stained with sodium phosphotungstate for 30 s and air-dried and then were observed using transmission electron microscopy (HT7800, Hitachi, Japan). The exosome markers CD63 and CD81 were analyzed by using western blot. Moreover, the size of exosomes was measured by nanoparticle tracking analysis (NTA) (RiboBio Ltd., Guangzhou, China).

2.4. Skin Wound-Healing Model and Treatment. Skin-defective mice were established ($n = 6$) as previously described [15]. Briefly, following anesthesia and hair shaving at the dorsal surface, a 1.2 cm diameter full-thickness skin excision was created on the back of the mice. Meanwhile, the mice were randomly divided into group A (BMMSC group, injection, 2×10^6 cells/mL), group B (JMMSC group, injection, 2×10^6 cells/mL), and the control group C (phosphate-buffered saline (PBS) group, injection, 200 μL). Then, we established the macrophage-depleted mice model (M-) ($n = 6$) through CL (clodronate liposomes, Nico van Rooijen lab, Holland) intravenous administration (5 mg/mL, 200 μL), then injected MSCs after 48 h and randomly divided them

into group D (BMMSC (M-) group, injection, 2×10^6 cells/mL) and group E (PBS (M-) group, injection, 200 μL). In order to maintain macrophage depletion, the CL was injected after every three days. In the exosome treatment experiment, skin-defective mice were established ($n = 4$) and randomly divided into group A (PBS group, injection, 200 μL), group B (BMMSC group, injection, 2×10^6 cells/mL), group C (BMSC-derived exosomes, injection, 200 μg), and group D (siRab27a interfered BMMSCs, injection, 2×10^6 cells/mL). Wound area was observed daily, and the wound-healing rate was calculated at different time points (days 3, 6, 9, and 12). The schemes for the description of the *in vivo* study are shown in Supplementary Figure S1. Wound areas were measured by tracing the wound margin and calculated using an image analysis program (ImageJ 1.48, National Institutes of Health). After the sacrifice of mice at the indicated time points, wound bed biopsies were divided into two parts for paraffin-embedded and frozen sections.

For MSCs labeled with CM-DIL (Thermo Fisher Scientific, Waltham, MA USA), which is a fluorescent dye well suited for monitoring cell movement or location and injected into the vein of the tail to explore the target cells of "homing" MSCs in the wound area, the adjacent normal skin was used as the control ($n = 3$). Mice were sacrificed on day 7 after treatment and skin samples were harvested for further analysis.

2.5. Histological and Immunohistochemistry Staining. The wound skin and surrounding skin were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4 μm sections. Standard HE staining and Masson trichrome staining were performed. To investigate the polarization of M2 macrophages *in vivo* and *in vitro*, indirect immunofluorescence studies of CD68 (sc-9139, 1 : 200), resistin-like molecule-(RELM-) α (sc-16120, 1 : 200), and CD14 (sc-9150, 1 : 200) and CD163 (sc-18796, 1 : 200, Santa Cruz Biotechnology, Dallas, Texas, USA) were performed as previously described [4]. Immunohistochemical analysis for CD31 (Abcam, ab28364, 1 : 20) and PCNA (Abcam, ab2426, 1 : 200) was performed as previously described [16]. The secondary antibodies, including donkey anti-rabbit IgG-FITC, Alexa Fluor 594 AffiniPure Donkey Anti-Goat IgG (H+L), and Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L), were purchased from Jackson ImmunoResearch Laboratories. For semi-quantification, positive signals from at least five random high-power fields were visualized, counted, and expressed as a percentage of total DAPI-positive cells (mean ± SD).

2.6. Coculture of MSCs or MSC-Derived Exosomes with Macrophages. For coculture studies, the CD14-positive monocytes were seeded into 6-well plates in RPMI 1640 media supplemented with 10% FBS, and on day 7, 2×10^5 BMMSCs or JMMSCs were seeded into the 0.4 μm pore size Transwell inserts (Millipore, Darmstadt, Germany) in an α-MEM containing 10% FBS, cocultured with macrophages for another 3 days. To study the function of MSC-derived exosomes on macrophage polarization, 2×10^5 BMMSCs, 50 μg/mL MSC-derived exosomes, and 2×10^5 BMMSCs transfected with siRab27a for 48 h were seeded with human PBMC-derived macrophages on day 7 and cultured for another 3 days. Then, macrophages were processed for the flow cytometric analysis (CytoFLEX, Beckman Coulter, CA, USA) of cell surface marker CD206 (BioLegend, San Diego, CA, USA), and the expression of CD206 was analyzed (R&D Systems, Minneapolis, Minnesota, USA) using immunofluorescence and RT-PCR.

2.7. Macrophages Uptake MSC-Derived Exosomes. MSC-derived exosomes were labeled with PKH26 (Sigma-Aldrich, St. Louis, MO, USA), as previously described with minor modification [17]. Human peripheral blood PBMC-derived macrophages, on day 7, were previously cultured with PKH26-labeled exosomes for 24 h at 37°C under 5% CO_2. After incubation, macrophages were washed twice with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. The sample was then washed twice with PBS and labeled with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Macrophage uptake of MSC-derived exosomes was observed under confocal laser microscope (Zeiss, Oberkochen FV1000, Germany).

2.8. Small Interfering RNA and Transfection Assays. For siRNA inhibition studies, MSCs were grown to 60% confluence followed by serum starvation for 12 h. siRab27a and a negative control (Santa Cruz Biotechnology, Dallas, Texas, USA) were transfected into cells at a final concentration of 50 nM using the Lipo2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection, the cells were harvested at 48 h for protein extraction.

For microRNA studies, MSCs were transfected with the miR-223 mimic at a final concentration of 50 nM and miR-223 inhibitor at a final concentration of 100 nM using the Lipo2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection, supernatant fractions collected from 48-hour cultures were used to isolate exosomes.

2.9. Western Blot Analysis. Cell lysates or mice skin homogenates were extracted using lysis buffer (10 mM Tris–HCl, 1 mM ethylene diamine tetra acetic acid (EDTA), 1% sodium dodecyl sulfate, 1% Nonidet P-40, 1 : 100 proteinase inhibitor cocktail, and 50 mM β-glycerophosphate, and 50 mM sodium fluoride) (Beyotime, Shanghai, China). The protein concentration was determined with a protein assay kit (Beyotime, Shanghai, China), following the manufacturer's instructions. Aliquots of 40 to 50 μg per sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked with 5% bovine serum albumin (BSA) in PBST (PBS with 0.1% Tween). Then, they were incubated with the following primary antibodies overnight: RELM-α (Santa Cruz Biotechnology, Dallas, Texas, USA), CD63 (Santa Cruz Biotechnology, Dallas, Texas, USA), CD81 (Santa Cruz Biotechnology, Dallas, Texas, USA), Rab27a (Abcam, Cambridge, UK), pknox1 (Abcam, Cambridge, UK), and anti-GAPDH (Abcam, Cambridge, UK). Then, the

membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Boster, Wuhan, China). The blots were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's instructions.

2.10. Total RNA Extraction and Quantitative RT-PCR. Total cellular RNA was extracted using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Isolated total RNA was then subjected to reverse transcription using Oligo dT primer and PrimeScript® RTase (Takara, Dalian, China), according to the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed with SYBR® Premix Ex Taq™ II (Takara, Dalian, China) using the C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The expression levels of the target genes were normalized to that of the housekeeping gene, GAPDH. The sequences of primers used are shown in Supplementary Table 1.

2.11. Statistical Analysis. All *in vitro* experiments were performed in triplicates with three different groups. The values were shown as the mean ± standard deviation (SD). The statistical differences between two groups were determined using two-tailed unpaired Student's *t*-test while those for more than two groups were determined using one-way Analysis of Variance (ANOVA) with the Bonferroni correction. All statistical analyses were done using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA), and P values less than 0.05 were considered statistically significant.

3. Results

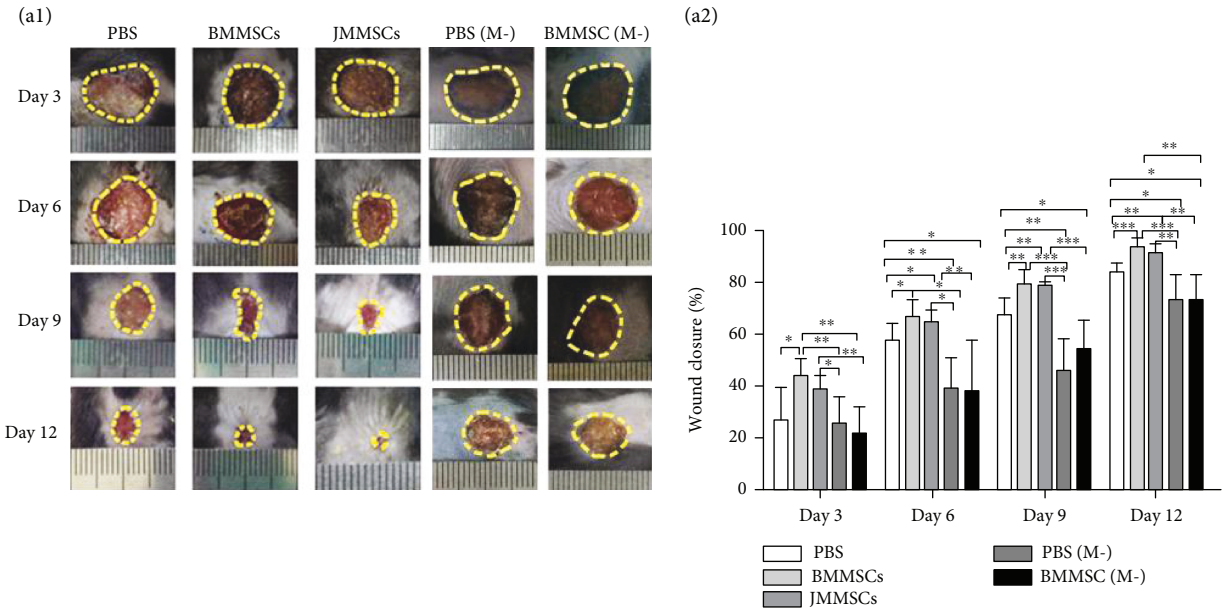
3.1. MSC-Based Therapy Is Macrophage-Dependent and Promotes Cutaneous Wound Healing. BMMSCs and JMMSCs were characterized for surface markers, osteogenesis, and adipogenesis (Supplementary Figure S2). To investigate the roles of MSCs in wound healing, two kinds of MSCs were systemically infused into mice 1 day post full-thickness skin excision and wound closure was carefully assessed after every three days ($n = 6$). Our results showed that mice that were infused with both kinds of MSCs exhibited accelerated skin wound closure compared with the control mice infused with only PBS (Figures 1(a) and 1(b)). The enhancement in wound closure appeared on day 3, and the wound became completely closed on day 12, which was as efficient as that shown by a previous study on the effect of gingiva-derived MSCs on promoting wound closure [4]. Moreover, to determine the role of macrophages in MSC-based therapy, we depleted macrophages in the early stage (Supplementary Figure S3) and observed the wound closure of mice with and without BMMSC therapy. Early depletion of macrophages significantly delayed the wound closure compared with that in the PBS, BMMSC infusion, and JMMSC infusion groups (Figures 1(a) and 1(b)), which indicated that macrophages were required in wound healing and that MSC therapy did not rescue the phenotype induced by macrophage depletion.

Collagen formation was evaluated in the form of a collagen index according to the previous report [18]. Masson trichrome staining showed a higher degree of collagen formation in the BMMSC and JMMSC treatment groups than in the PBS group and the macrophage depletion groups (Figure 1(c)). In addition, to further determine the effects of MSC on wound healing, we stained the vascular endothelial marker, CD31, and proliferative marker, PCNA, in the wound bed area. We discovered that the percentage of the CD31 and PCNA positively stained area increased upon BMMSC or JMMSC treatment as compared with that in the PBS group and the macrophage depletion groups (Figures 1(d) and 1(e)). These findings indicated that BMMSC or JMMSC treatment might lead to more prominent effects on angiogenesis and cell proliferation during wound healing.

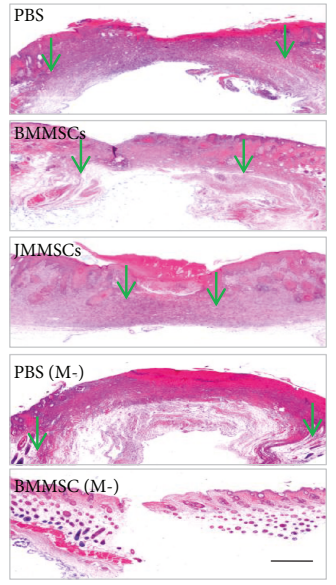
3.2. Systemically Infused MSCs Home to the Wound Site and Skew Macrophages to M2. In order to investigate the *in vivo* interaction of MSCs and host macrophages, BMMSCs and JMMSCs, prelabeled with CM-DiL, were systemically injected into mice ($n = 3$). The numbers of BMMSCs and JMMSCs that home to the wound site were increased compared with the cell numbers in the same site of normal skin (Supplementary Figure S4a). However, there was no significant differences between the numbers of BMMSCs and JMMSCs that were homing to the wound site (Supplementary Figure S4a). In addition, BMMSCs and JMMSCs were in close proximity with CD68-positive macrophages at the wound site (Supplementary Figure S4b).

We next explored the *in vivo* effects of BMMSCs and JMMSCs on the phenotype of macrophages located at the wound area. Macrophages were stained using dual-color immunofluorescence, specific antibodies for CD68 (green), and RELM-α (red). CD68 is a surface marker of macrophage [19, 20]. RELM-α is a well-known marker for M2 macrophages [21, 22] and macrophages showing the wound-healing phenotype [23]. After infusion of BMMSCs and JBMMSCs, the time-dependent increase in the number of both RELM-α and CD68-positive cells (yellow) was observed (Figure 2(a)). We also verified that systemic injection of BMMSCs and JMMSCs could promote the RELM-α expression at the wound site, but not in the normal skin (Figure 2(b)).

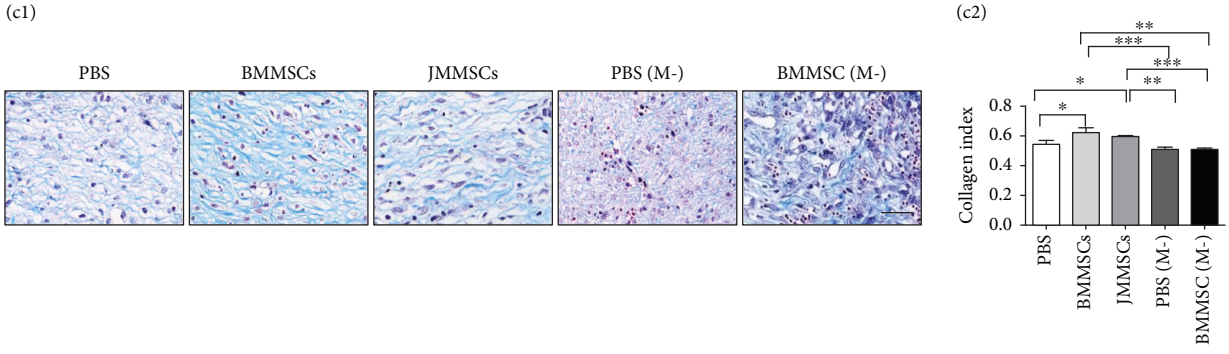
To further investigate whether BMMSCs and JMMSCs convert macrophages into those with the M2 phenotype, human PBMC-derived macrophages were, respectively, cocultured with BMMSCs or JMMSCs at a ratio 1 : 2.5~1 : 5 for 72 h in the Transwell system. Then, macrophages were stained with CD14 and CD163, which is an M2a marker, induced by IL-4 or IL-13 and associated with tissue repair [24]. The results showed a higher number of CD14 and CD163 double-positive macrophages (Figure 2(c)) after coculturing with MSCs. In addition, the expression of CD206, a marker of an M2 and wound-healing macrophage [23], and HLA-DR [25], one of the markers for M1 macrophages, was assessed in $CD14^+$ macrophages after coculturing with BMMSCs or JMMSCs using flow cytometry. The results showed a higher number of CD206 macrophages in



(a)



(b)



(c)

Figure 1: Continued.

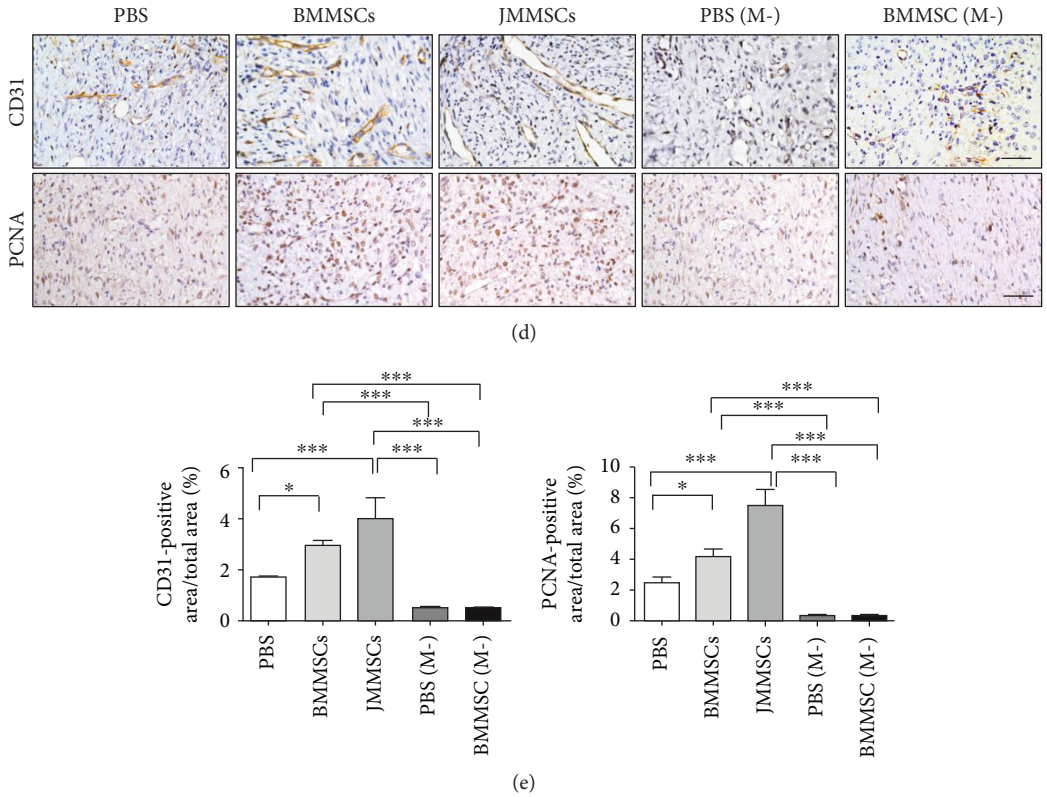


Figure 1: MSC-based therapy is macrophage-dependent and enhances cutaneous wound healing. (a) Representative light field photographs of cutaneous wounds in normal mice or macrophage-depleted mice after treatment with PBS, BMMSCs or JMMSCs (a1); the whole cutaneous wound is outlined in a dashed line. Percentage of the wound closure on day 3 to day 12 in reference to the day 0 wounds from the groups described in the left figures (a2) ($n = 6$). (b) Representative H&E image from a cutaneous wound at day 12, the green arrows indicating the wound edge. (c) Masson trichrome (c1) showing collagen deposition at day 12 and quantification of collagen index (c2) ($n = 3$). (d) Immunostaining of CD31 and proliferating cell nuclear antigen (PCNA) at day 12 of skin wound ($n = 3$). (e) Quantification of immunostaining of CD31 and PCNA positively stained area percentages at day 12 of skin wound ($n = 3$). Scale bars: 500 μm (b) and 50 μm (c, d). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Error bars are mean ± SD.

BMMSC or JMMSC group compared to the control group (Figure 2(d), Supplementary Figure S5). However, there was no significant difference in expression of HLA-DR among the three groups (Supplementary Figure S5). The macrophages also expressed higher levels of IL-10 and lower levels of TNF-α after coculturing with BMMSCs or JBMMSCs compared to the control group (Figure 2(e)). Taken together, these results elucidated the positive effects of BMMSCs or JMMSCs in inducing M2 polarization of macrophages both *in vivo* and *in vitro*.

3.3. Uptake of MSC-Secreted Exosomes by Macrophages Promotes M2 Polarization. These findings led us to investigate which factors participate in MSC-induced polarization of M2 macrophages. Next, we isolated exosomes secreted by BMMSCs (BMMSC-ex) or JMMSCs (JMMSC-ex) and observed them using transmission electron microscopy (TEM). Exosomes exhibited a cup-shaped morphology, as shown by TEM (Supplementary Figure S6a). Nanoparticle tracking analysis (NTA) revealed that the isolated exosomes from BMMSCs possessed diameters ranging from 20 to 200 nm, with a mean diameter of 27.46 nm (Supplementary Figure S6b), and the exosomal markers, such as CD63 and CD81, were examined in BMMSC-ex and JMMSC-ex (Figure 3(a)). We added the PKH26-labeled BMMSC-ex or JMMSC-ex into the macrophage cultures, and after 24 h, the PKH26-labeled exosomes were observed in macrophages. However, PBS group cells did not exhibit any red fluorescence (Figure 3(b)). We also collected the supernatant of BMMSCs and JMMSCs and measured the total amount of exosome protein purified from culture medium (Supplementary Figure S6c). Western blot analysis also showed that BMMSCs and JMMSCs expressed Rab27a (Supplementary Figure S6d), which regulated the release of exosomes [25].

To know whether exosomes are involved in BMMSC-mediated polarization of M2 macrophages, we used Rab27a siRNA to decrease exosome secretion of BMMSCs. Firstly, the expression of Rab27a was downregulated after BMMSCs were transfected with Rab27a siRNA (BM/siRab27a, Supplementary Figure S6e). Then, the exosome secretion was inhibited after Rab27a knockdown (Supplementary Figure S6f). After that, BMMSCs, BMMSC-ex, and BM/siRab27a were added to the culture medium of macrophages. Macrophages without coculture were used as the control. The results showed that the percentage of CD206-positive cells was increased in the

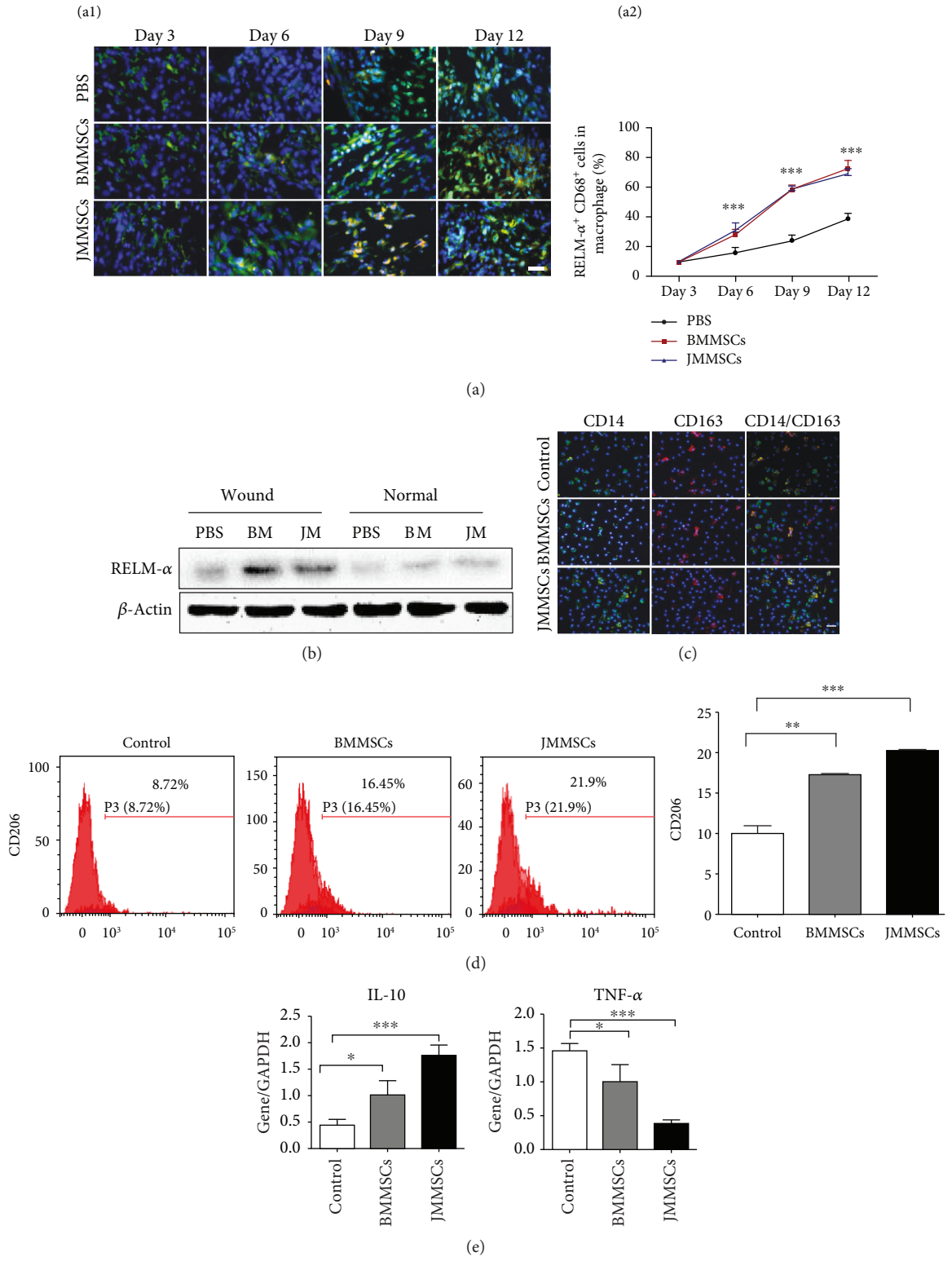
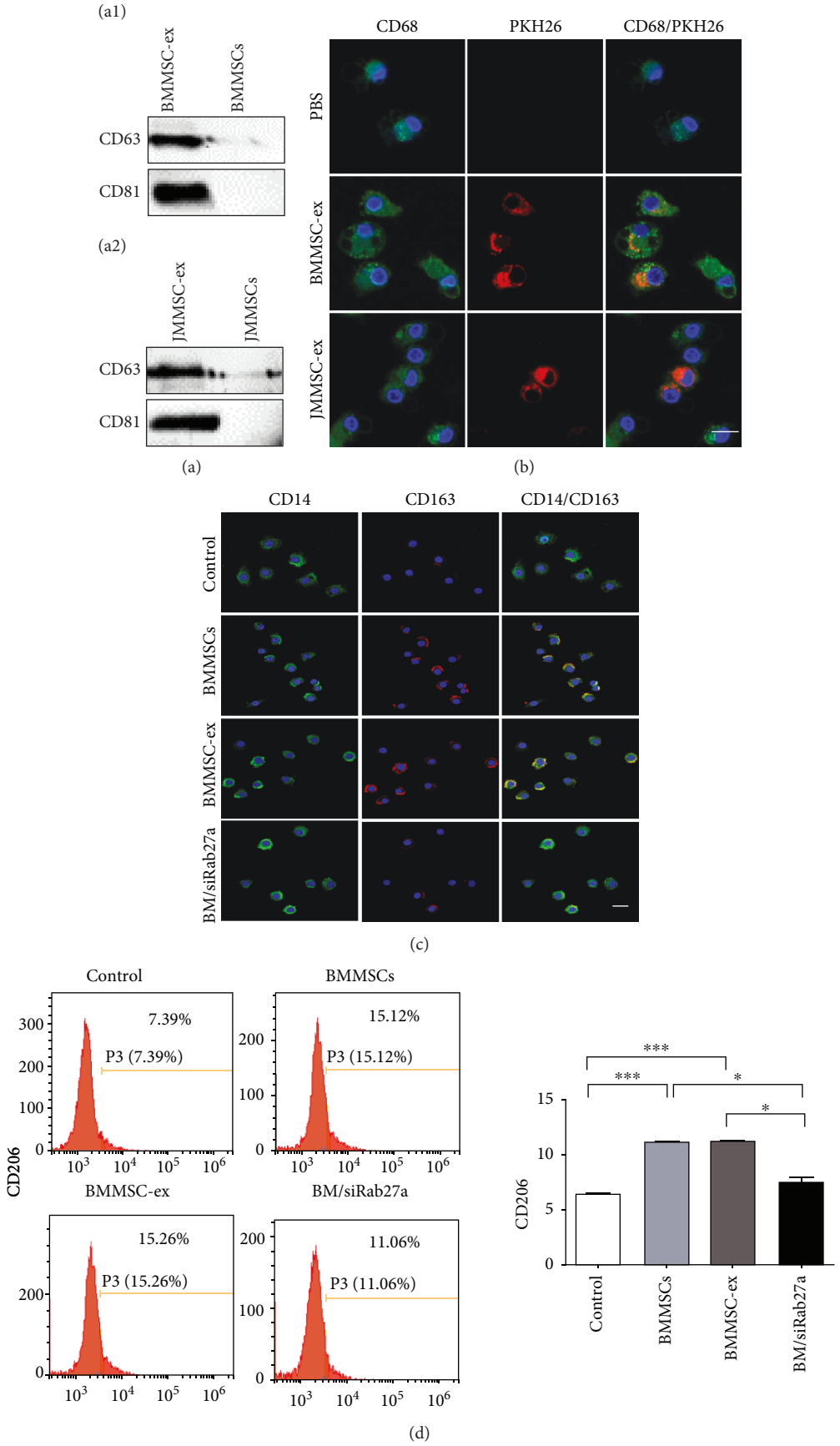


Figure 2: Systemically infused MSCs skew macrophages to M2. (a) Dual-color immunofluorescence staining of CD68 (green) and RELM-α (red) at the wound site after systemic injection of BMMSCs and JMMSCs at days 3, 6, 9, and 12 (a1). Cell nuclei were counterstained with DAPI (blue). Comparison of the percentage of RELM-α and CD68 dual-positive macrophages (a2) ($n = 3$). (b) Western blot analysis of RELM-α expression in the wound samples and the surrounding normal skin samples after systemic injection of BMMSCs and JMMSCs. (c) Dual-color immunofluorescence staining of CD14 (green) and CD163 (red) in macrophages after being cocultured with BMMSCs or JBMMSCs. (d) The percentage of CD206-positive cells in macrophages after coculture with BMMSCs or JMMSCs by flow cytometry ($n = 3$). (e) qRT-PCR analysis of IL-10 and TNF-α in macrophages after being cocultured with BMMSCs or JBMMSCs ($n = 3$). Scale bars: 100 μm (a, c). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Error bars are mean ± SD.



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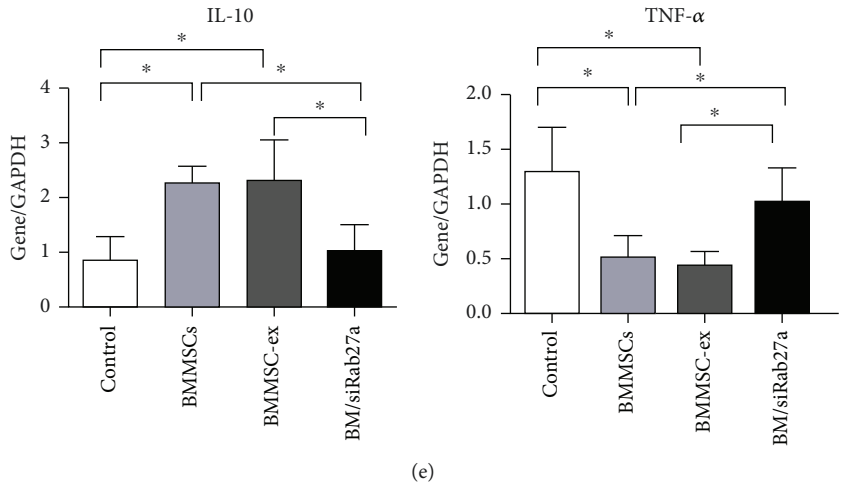


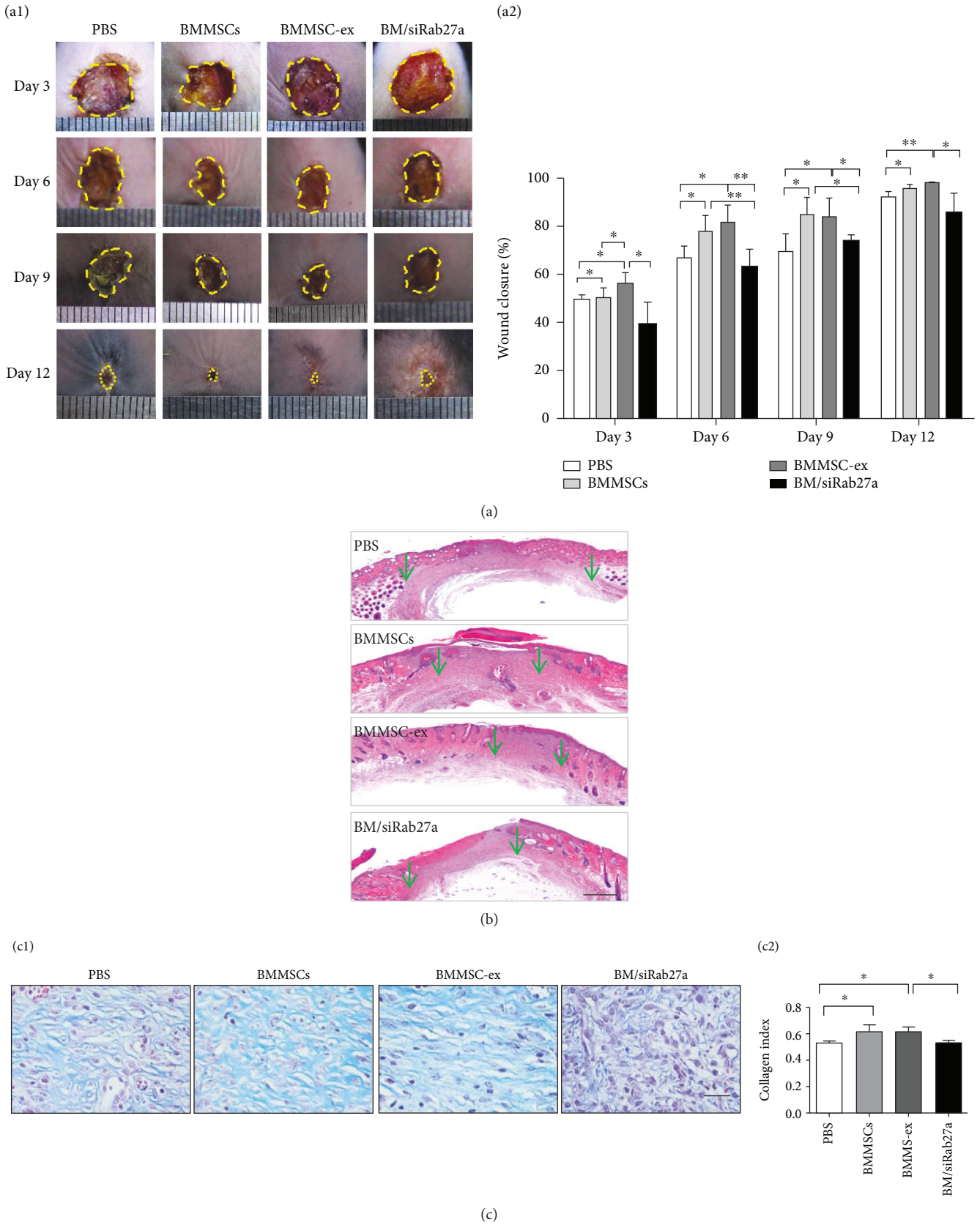
Figure 3: Uptake of MSC-secreted exosomes by macrophages promotes M2 polarization. (a) The expression of CD63 and CD81 in BMMSC-ex and BMMSCs (a1) and JMMSC-ex and JMMSCs (a2) assessed by western blot analysis. (b) Exosomes (PKH26, red) from BMMSCs or JBMMSCs entered into macrophages (CD68, green). (c) CD14 (green) and CD163 (red) staining of macrophages after being cocultured with BMMSC-, BMMSC/siRab27a-, or BMMSC-derived exosomes. Macrophages cocultured with BM/siRab27a showed less CD14 and CD163 double-positive cells compared to the BMMSC or BMMSC-ex group. (d) CD206-positive macrophages after being cocultured with exosomes and BMMSCs were assessed by flow cytometric analysis ($n = 3$). CD206 expression increased compared with the macrophages without treatment. However, the number of CD206-positive macrophages decreased after coculture with BMMSCs of Rab27a knockdown compared to the BMMSC and BMMSC-ex groups. (e) qRT-PCR analysis of IL-10 and TNF-α in macrophages after being cocultured with BMMSC-, BMMSC/siRab27a-, or BMMSC-derived exosomes ($n = 3$). Macrophages cocultured with BM/siRab27a showed lower IL-10 and higher TNF-α compared to the BMMSC or BMMSC-ex group. Scale bars: 50 μm (b, c). $^{*}P < 0.05$ and $^{***}P < 0.001$. Error bars are mean ± SD.

three groups after coculturing with BMMSCs, BMMSC-ex, and BM/siRab27a compared to the control group (Figure 3(d)). However, BM/siRab27a decreased the M2 polarization of macrophages compared to the BMMSC and BMMSC-ex groups (Figure 3(d)). The immunofluorescence staining of CD14 and CD163 showed a higher number of CD14 and CD163 double-positive cells after coculturing with BMMSCs or BMMSC-ex compared to the control group (Figure 3(c)). Macrophages cocultured with BM/siRab27a showed a lower number of CD14 and CD163 double-positive cells compared to the BMMSC or BMMSC-ex groups (Figure 3(c)). Compared with the control group, macrophages expressed a higher level of IL-10 and a lower level of TNF-α after coculturing with BMMSCs or BMMSC-ex. However, there was no significant difference in the expression of either IL-10 or TNF-α in macrophages cocultured with BM/siRab27a (Figure 3(e)).

3.4. MSCT Enhances Cutaneous Wound Healing and Skews Macrophages to the M2 Phenotype through Exosomes. Next, we investigated the *in vivo* effects of exosomes secreted by BMMSCs on wound repair and M2 polarization. BMMSCs, BMMSC-ex, and BM/siRab27a were systemically infused into mice 1 day post full-thickness skin excision, and wound closure was carefully assessed after every three days ($n = 4$). As shown, the mice that received BMMSC and BMMSC-ex infusion had substantially accelerated cutaneous wound healing, while BM/siRab27a infusion delayed wound healing at days 3, 6, 9, and 12 (Figures 4(a) and 4(b)). Masson trichrome staining also showed a higher degree of collagen formation in the BMMSC or BMMSC-ex treatment groups compared to the PBS and BM/siRab27a groups (Figure 4(c)). In addition, a higher proportion of the CD31 and PCNA positively stained area was observed in BMMSC- or BMMSC-ex-treated wounds as compared with the PBS and BM/siRab27a groups (Figures 4(d) and 4(e)). These results demonstrated the promoting effects of BMMSC-derived exosomes on cutaneous wound healing.

Further analysis on the CD68 and RELM-α double-positive cells at the wound site confirmed the positive roles of exosomes on the M2 polarization of macrophages. The results showed that the number of CD68 and RELM-α double-positive cells were increased in the BMMSC and BMMSC-ex groups compared to the PBS and BM/siRab27a groups (Figure 5(a)). In addition, western blot assay of the wound site tissue showed similar effects, in that the expression of RELM-α was increased in the BMMSC and BMMSC-ex groups (Figure 5(b)). Moreover, expression of Arg-1 in the wound site was increased and expression of TNF-α was decreased in the BMMSC and BMMSC-ex groups compared to the PBS and BM/siRab27a groups as shown by qRT-PCR analysis (Figure 5(c)).

3.5. MSCs Skew Macrophages to the M2 Phenotype via Transferring Exosome-Derived miR-223. miR-223 has been previously reported to promote macrophages to the M2 phenotype [26]. Collino et al. [27] reported that miR-223 was expressed in MSCs. Therefore, we first examined whether MSCs transferred miR-223 to macrophages. After coculturing with BMMSCs and BMMSC-ex, we investigated the expression of miR-223 in macrophages. The results showed that the expression of miR-223 was increased in



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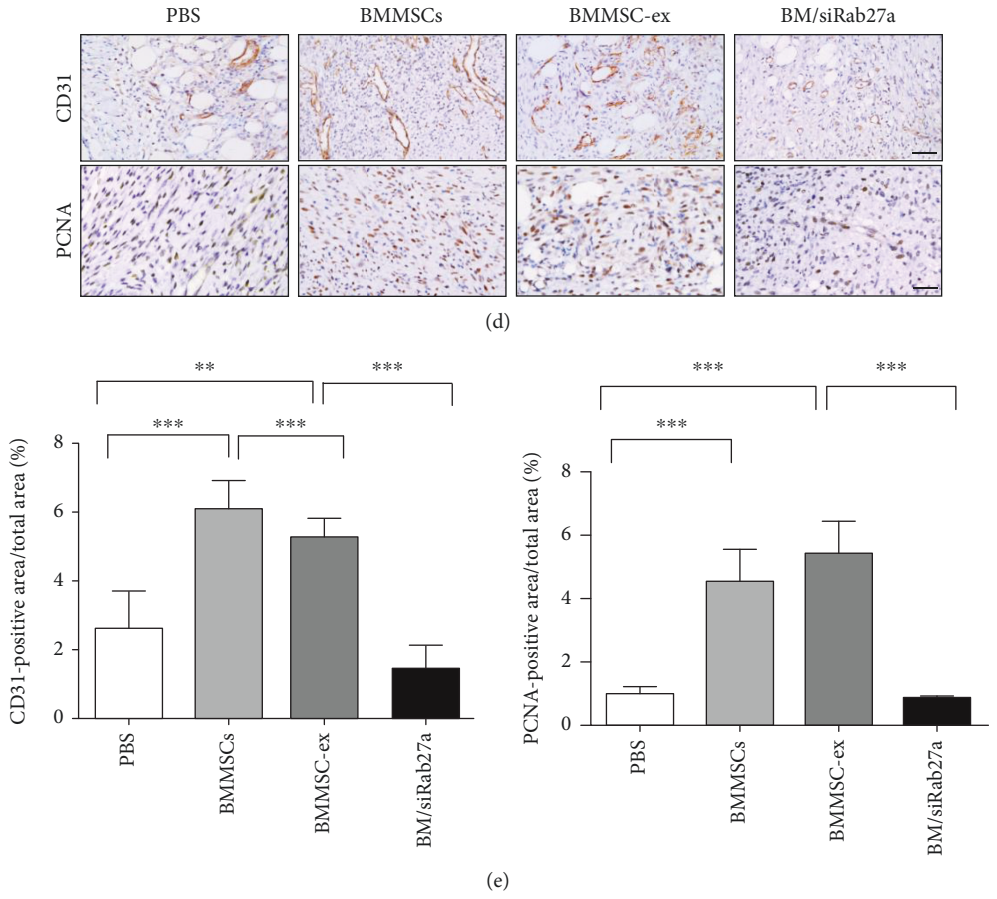

Figure 4: MSCs enhance wound healing through exosomes. (a) Representative light field photographs of cutaneous wounds after treatment with PBS, BMMSCs, BMMSC-ex, and BM/siRab27a (a1); the whole cutaneous wound is outlined in a dashed line. Percentage of the wound closure on day 3 to day 12 in reference to the day 0 wounds (a2) ($n = 4$). BMMSC- and BMMSC-ex-treated wounds showed a statistically significant increase in wound closure comparing with the PBS-treated wounds, whereas BM/siRab27a-treated wounds had no significant difference comparing with the PBS group at different time points. (b) Representative H&E image from a cutaneous wound at day 12, the green arrows indicating the margin of wound-healing area. (c) Masson trichrome (c1) showing collagen deposition at day 12 and quantification of collagen index (c2) ($n = 3$). (d) CD31 and PCNA staining of the skin wound at day 12. (e) Quantification of immunostaining of CD31- and PCNA-positive cells at day 12 of the skin wound ($n = 3$). Scale bars: 500 μm (b) and 50 μm (c, d). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Error bars are mean ± SD.

macrophages cocultured with BMMSCs or BMMSC-ex compared to macrophages not cocultured (Figure 6(a)). Then, we used miR-223 mimics or inhibitors to overexpress or inhibit the miR-223 expression in BMMSCs, respectively (Figure 6(b)), and we investigated the expression of miR-223 in exosomes secreted by BMMSCs. miR-223 expression was profoundly inhibited and promoted after transfection with miR-223 inhibitors and mimics, respectively (Figure 6(c)). To determine whether miR-223 regulates M2 polarization of macrophages, we detected the CD206 expression of macrophages after culturing with exosomes, in which miR-223 was overexpressed or knocked down. Flow cytometry analysis showed a higher number of CD206-positive macrophages in the miR-223 mimic group and less number of CD206-positive macrophages in the miR-223 inhibitor group compared to those cultured with exosomes without treatment (Figure 6(d)). Considering pknox1 is a validated target gene of miR-223, we detected whether miR-223 in exosomes suppresses the pknox1 protein level in macrophages after coculturing. As anticipated, western blot assays revealed that overexpression of miR-223 significantly diminished accumulation of the pknox1 protein, whereas knockdown of miR-223 elevated pknox1 protein levels (Figure 6(e)). Taken together, these results showed that exosome-derived miR-223 may be an important factor to promote macrophages to the M2 phenotype.

4. Discussion

During the wound-healing process, immune cells reside in the wound site where they regulate inflammation and mediate the tissue repair [28]. Despite entrapment of intravenously injected MSCs in the lung, they are still capable of migrating to the site of inflammation and injury [29]. MSCs exert their immunomodulatory properties by regulating the function of both innate and adaptive immune cells via mechanisms involving both direct cell-cell contact and/or soluble factors [12, 13, 30, 31]. MSCs can play an important role in

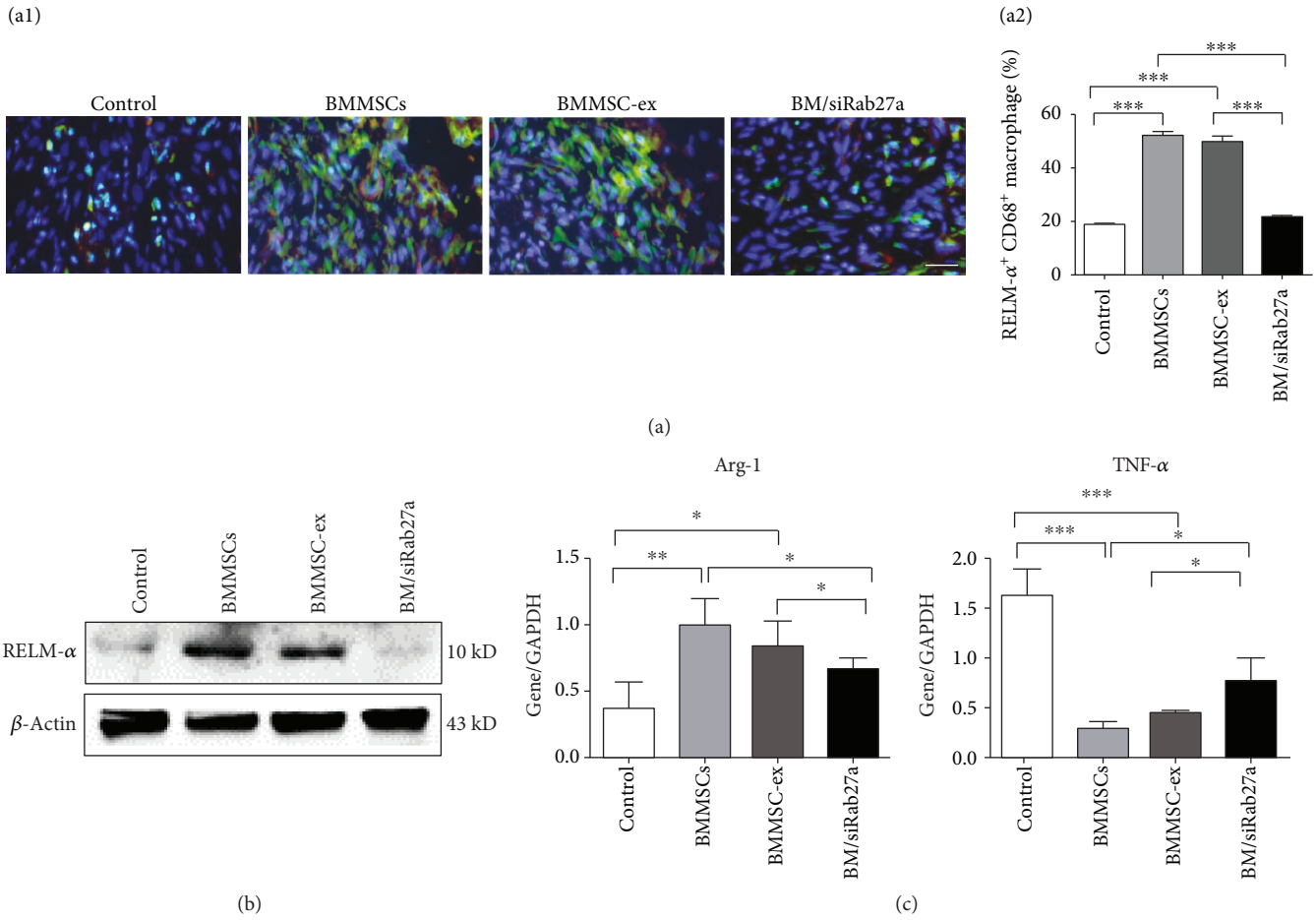

Figure 5: MSCs skew macrophages to M2 through exosomes. (a) Immunofluorescence staining of CD68 (green) and RELM-α (red) at the wound site after systemic injection of BMMSC-, BMMSC/siRab27a-, or BMMSC-derived exosomes (a1); the percentage of M2 in $CD68^+$ macrophages (a2) ($n = 3$). (b) Western blot analysis of RELM-α expression at the wound site. RELM-α expression was increased in the BMMSC and BMMSC-ex groups compared to the PBS and BM/siRab27a groups. (c) qRT-PCR analysis of Arg-1and TNF-α at the wound site. A high level of TNF-α and a low level of Arg-1 were detected in the PBS and BM/siRab27a groups compared to the BMMSC and BMMSC-ex groups ($n = 3$). Scale bar: 100 μm (a).$^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Error bars are mean ± SD.

the wound-healing process via the secretion of soluble factors, such as TGF-β1 [32] and TSP-1 [33]. However, cell-cell interaction after MSCT that promotes skin repair still remains unclear.

The inflammatory response is a crucial component of cutaneous wound healing, as evidenced by severely delayed repair following *in vivo* macrophage ablation [6]. In response to signals derived from the injury, macrophages undergo a reprogramming that leads to the emergence of a spectrum of distinct functional phenotypes. Depending on the cytokines IFN-γ and TNF-α, M1 macrophages upregulate the enzyme-inducible nitric oxide synthase (iNOS) and produce a variety of proinflammatory cytokines, including IL-1, IL-6, and IL-23. Conversely, M2 macrophages, dependent on IL-4 and IL-13, released from T_H2 lymphocytes in response to tissue injury, upregulate the enzymes Arginase 1, Fizz, and Ym1 [34]. A study by Chen et al. [35] found that MSCs can promote macrophage M2 polarization by secreting TGF-β3 and TSP1. Growing evidence has shown that M2 macrophages resolve the inflammation and promote wound healing [7, 8]. Human gingiva-derived MSC transplantation enhanced cutaneous wound healing by inducing M2 polarization of macrophages at the wound site [4]. In our study, we also found that MSCT promotes M2 polarization of macrophages at the wound site. The expression of M2-specific factors, such as RELM-α and Arginase 1, was increased at the wound site. Furthermore, MSCs also induced M2 macrophage differentiation *in vitro*. The macrophages expressed higher levels of IL-10 and lower levels of TNF-α after coculturing.

Exosomes contain several molecules, such as proteins and miRNAs, and serve as a new mechanism for cell-cell communication [36, 37]. Tumor-derived exosomes are important tumorigenesis mediators capable of inducing neoplastic transformation and tumor metastasis in stromal/stem cells [38, 39]. Meanwhile, stromal cell-derived exosomes promote cancer cell migration [40]. These evidences suggested that exosomes mediate the crosstalk between tumor cells and surrounding stromal cells. Recently, increasing amount of evidence of the therapeutic potential of MSC-derived exosomes in promoting cutaneous wounding healing has emerged [12, 41]. Exosomes derived from human umbilical

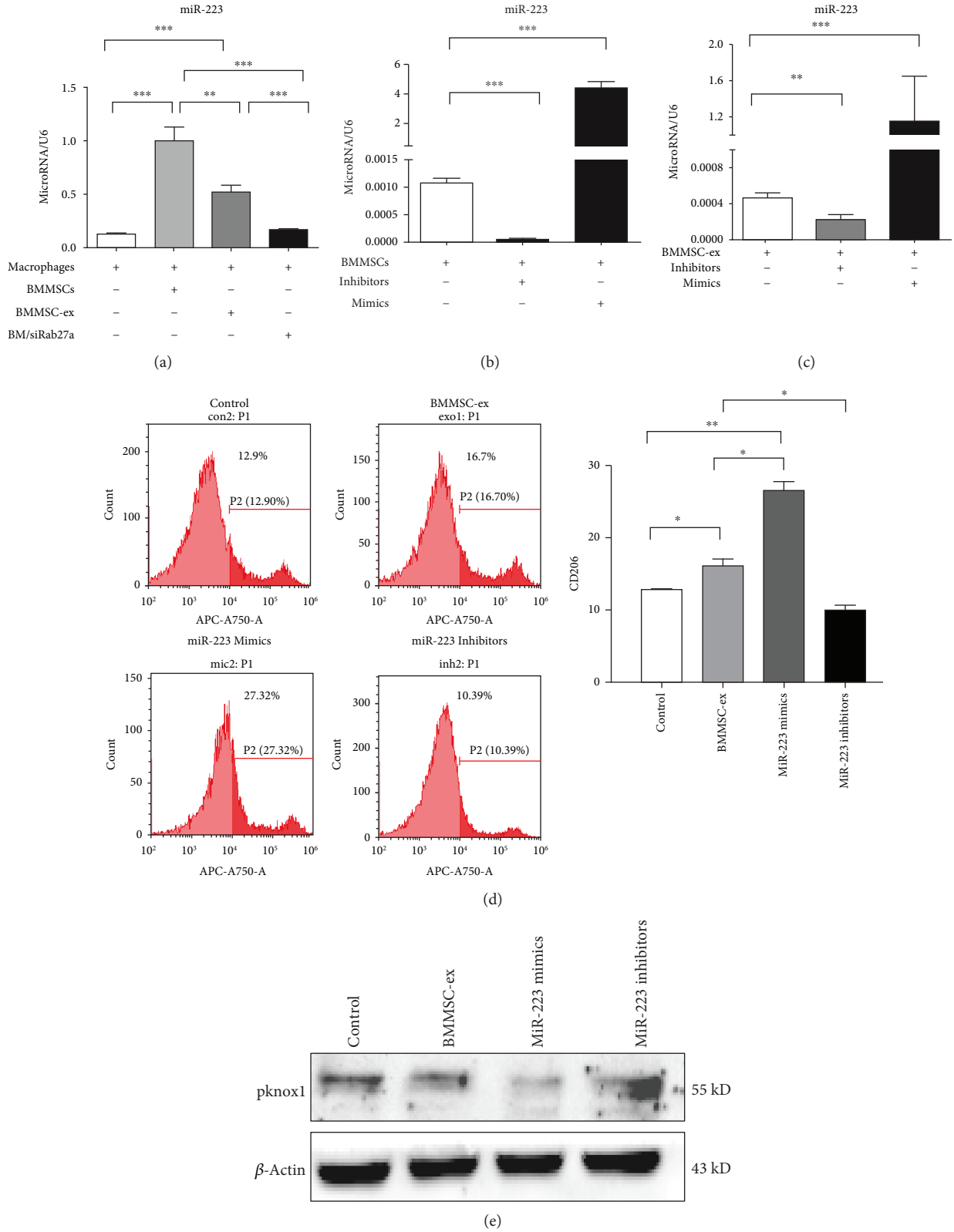

Figure 6: MSCs skew macrophages to M2 via transferring exosome-derived miR-223. (a) qRT-PCR analysis of miR-223 in macrophages cocultured with BMMSCs, BMMSC-ex, and BM/siRab27a. (b) Analysis of miR-223 in BMMSCs transfected with miR-223 mimics and inhibitors. (c) Analysis of miR-223 in exosomes derived from BMMSCs transfected with miR-223 mimics and inhibitors. (d) CD206-positive macrophages were assessed after being cocultured with exosomes derived from BMMSCs, which were transfected with miR-223 mimics or inhibitors ($n = 3$). (e) Western bolt analysis of pknox1 in macrophages after being cocultured with exosomes derived from BMMSCs, which were transfected with miR-223 mimics or inhibitors. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Error bars are mean ± SD.

cord MSCs enhance proliferation and migration of skin cells via Wnt4-mediated β-catenin nuclear translocation [12]. In our study, when we inhibited the secretion of exosomes in MSCs, the number of M2 macrophages was decreased both in the *in vitro* coculture system and in the *in vivo* interaction site. These results indicated that MSCT can elicit M2 polarization of macrophage by secreting exosomes.

Exosomes have been demonstrated to play an important role in skin wound healing; however, to our knowledge, only a few studies have reported the effects of MSC-derived exosomes on M2 polarization of the macrophage. Exosomes contain microRNA and are involved in intracellular communication. We revealed that exosomes secreted by MSCs contained miR-223, which contributed to macrophage polarization. miR-223, which suppresses classic proinflammatory pathways and enhances the alternative anti-inflammatory responses, is a novel regulator of macrophage polarization [26, 42, 43]. In addition, pknox1 is identified as a genuine miR-223 target gene and an essential regulator of macrophage polarization [44]. Here, we also found that knockdown of miR-223 in MSCs reduced M2 polarization of the macrophage. Alteration of the pknox1 expression was observed in the macrophage after coculturing with exosomes isolated from BMMSCs that were transfected with miR-223 mimics or inhibitors. Previous studies have showed that exosomes derived from LPS-preconditioned MSCs contained let-7b, which skewed M2 polarization of the macrophage [41, 45]. In addition, miR-146a has been reported to negatively regulate the wound healing in a diabetic murine wound-healing model [46]. We could not preclude the other miRNAs or factors contained in exosomes derived from MSCs that may induce M2 polarization during MSCT. MSCT may use multiple mechanisms to promote cutaneous wound healing, and further study is still needed to explore the other mechanisms of MSCT.

Taken together, our findings provided the evidence that MSCT elicits M2 polarization of macrophages and accelerates wound healing, in part, via transferring donor exosome-derived microRNA. Thus, the microRNAs of exosomes derived from MSCs could be a therapeutic target for cutaneous wound healing.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xiaoning He, Zhiwei Dong, and Yina Cao contributed equally to this work.

Acknowledgments

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Supplementary Materials

The supplementary material file is the supplementary figures associated with the results that might be of interest to readers. *(Supplementary Materials)*

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Review Article

Enhancing Mesenchymal Stromal Cell Immunomodulation for Treating Conditions Influenced by the Immune System

Bella S. Guerrouahen, Heba Sidahmed, Asma Al Sulaiti, Moza Al Khulaifi, and Chiara Cugno

Sidra Medicine, Member of Qatar Foundation, Doha, Qatar

Correspondence should be addressed to Bella S. Guerrouahen; bguerrouahen@sidra.org

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Mesenchymal stromal cells (MSCs), formerly known as mesenchymal stem cells, are nonhematopoietic multipotent cells and are emerging worldwide as the most clinically used and promising source for allogeneic cell therapy. MSCs, initially obtained from bone marrow, can be derived from several other tissues, such as adipose tissue, placenta, and umbilical cord. Diversity in tissue sourcing and manufacturing procedures has significant effects on MSC products. However, in 2006, a minimal set of standard criteria has been issued by the International Society of Cellular Therapy for defining derived MSCs. These include adherence to plastic in conventional culture conditions, particular phenotype, and multilineage differentiation capacity *in vitro*. Moreover, MSCs have trophic capabilities, a high *in vitro* self-renewal ability, and immunomodulatory characteristics. Thus, immunosuppressive treatment with MSCs has been proposed as a potential therapeutic alternative for conditions in which the immune system cells influence outcomes, such as inflammatory and autoimmune diseases. The precise mechanism by which MSCs affect functions of most immune effector cells is not completely understood but involves direct contact with immune cells, soluble mediators, and local microenvironmental factors. Recently, it has been shown that their homeostatic resting state requires activation, which can be achieved *in vitro* with various cytokines, including interferon-γ. In the present review, we focus on the suppressive effect that MSCs exert on the immune system and highlight the significance of *in vitro* preconditioning and its use in preclinical studies. We discuss the clinical aspects of using MSCs as an immunomodulatory treatment. Finally, we comment on the risk of interfering with the immune system in regard to cancer formation and development.

1. Background

Mesenchymal stromal cells (MSCs) are nonhematopoietic cells which possess self-renewal, proliferative, and clonogenic potential and have the ability to commit to different cell types including adipocytes, chondrocytes, and osteocytes depending on the environmental conditions [1–3]. They can be easily isolated from human tissues and have exceptional biological properties for advanced therapies [4]. Traditionally derived from bone marrow (BM) [5], MSC populations may also be obtained from other various tissue sources, such as maternal decidua basalis of the placenta, adipose tissue (AT), foreskin, or neonatal birth-associated tissues (fetal part of the placenta and umbilical cord (UC)) [6, 7]. In 2006, the International Society for Cellular Therapy (ISCT) established the minimum criteria for designating MSCs derived from various origins: adherence to plastic in standard culture conditions; expression of different nonspecific surface molecules such as CD105/endoglin, CD90/Thy1, and CD73/5′-nucleotidase; lack of expression of CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR (<2%); and trilineage differentiation potential due to the expression of several pluripotency genes. The weak expression of major histocompatibility complex (MHC) class I protects MSCs from natural killer (NK) cell-mediated killing; additionally, the lack of MHC class II expression confers to these cells the ability to evade immune recognition by $CD4^+$ T cells. MSCs present minimal expression for HLA-DR (<2%) and do not express costimulatory proteins (CD80, CD86, and CD40), endothelial or hematopoietic surface molecule markers, such as

CD31, CD45, CD34, CD14 or CD11b, and CD79a or CD19 [8]. New developments in characterization and marker profiling improve the methods of isolation, verification, and quality assessment of MSCs. In addition to hematopoietic support, tissue repair after injury, and use in regenerative medicine, the immunomodulatory properties of MSCs are attributes that represent the rationale for using MSCs as a novel therapy for many diseases, particularly disorders of the immune system [9–13]. Interestingly, the ISCT issued guidelines pertaining to MSC effector pathways such as immunomodulation, regeneration, and homing properties [14]. In 2002, for the first time, it was demonstrated that MSCs can modulate immunosuppression *in vitro* and *in vivo* [15]. For Caplan, the acronym MSC stands for "medicinal signaling cells," indicating that the main attribute of MSC therapy is the secretion of bioactive molecules (extracellular vesicles (EVs), cytokines, growth factors, and chemokines) [16], and Caplan and Correa later proposed that the trophic and immunomodulatory properties of MSCs may function as site-regulated "drugstores" *in vivo* [17]. MSCs were also called the "guardians of inflammation" [18]. Those properties confer the clinical value of MSCs through the interaction with immune cells and the secretion of bioactive molecules leading to the suppression of lymphocyte proliferation, maturation of monocytes, and generation of regulatory T cells (Tregs) and M2 macrophages [19, 20]. In this review, we focus on the immunomodulatory effects of MSCs, the value of preconditioning, and its application in preclinical studies. We then comment on some clinical trials using MSCs and encountered hurdles. Finally, we discuss the risk of modulating the action of immune cells, which might theoretically favor the formation and development of cancer.

2. MSC-Mediated Immunomodulation of Immune Cells

MSCs were described as sensors of the inflammatory microenvironment in regard to their impact on the immune system [21]. Through cell-to-cell contact and regulatory molecule secretion which includes growth factors, chemokines, cytokines, and EVs, MSCs regulate both innate and adaptive immunity by affecting the activation, maturation, proliferation, differentiation, and effector functions of T and B lymphocytes (adaptive immune system), NK cells, neutrophils, and macrophages (innate immune system), as well as dendritic cells (DC), which link innate to adaptive immunity [22, 23].

2.1. T Lymphocytes. Activated T cells proliferate and release inflammatory cytokines and chemokines [24]. In the inflammatory environment, MSCs recruit local helper (Th) and effector T cells, via highly expressed chemokine (C-X-C motif) ligands CXCL9 and CXCL10, thus facilitating their immunomodulatory activity [25]. The intracellular enzymes indoleamine-2,3-dioxygenase (IDO) and inducible NO synthase (iNOS) produced by MSCs are some of the major mediators of T cell suppression, prompting their polarity shift from a proinflammatory Th1 state to an anti-inflammatory Th2 condition [26–28]. Galectin-1, abundantly expressed in and secreted by MSCs, also acts on T lymphocyte subpopulations and influences their cytokine production and release [29]. Interleukin- (IL-) 10, transforming growth factor- (TGF-) β, and the lipid mediator prostaglandin E2 (PGE2) secretion by MSCs inhibit Th17 cell differentiation and inhibit the production of IL-17, IL-22, interferon- (IFN-) γ, and tumor necrosis factor- (TNF-) α by mature Th17 cells [30–33]. In addition, TGF-β enhances T regulatory cell (Treg) function and differentiation, thus collectively modulating the Treg/Th17 balance [32]. Besides, the Notch 1 signaling pathway has been involved in MSC-mediated Treg differentiation [34], and the IL-10-dependent secretion of HLA-G5 further expands the Treg compartment [35].

2.2. B Lymphocytes. B cells are indispensable for humoral immunity and secrete antibodies when stimulated by antigens and inflammatory cytokines such as IL-10. Under quiescent conditions, MSCs trigger the differentiation into regulatory B cells (Bregs) [36]; while during inflammation, MSCs inhibit B cell proliferation, dampen the production of immunoglobulins (IgA, IgG, and IgM), and lose the capacity to induce Bregs [36–38]. While the potential of MSCs in B cell immunomodulation is not fully understood, it appears that inflammatory conditions are necessary for MSCs to exert their role through a combination of cell-cell contact (e.g., PD-L1 pathway) and soluble factors [39, 40].

2.3. NK Cells. Considered a subset of lymphocytes, NK cells are an important source of IFN-γ in addition to T cells [41]. MSCs are able to dampen the expansion of NK cells, effector functions, and cytotoxic production through the key mediators PGE2, IDO, and HLA-G5 [35, 42, 43].

2.4. Neutrophils. During inflammatory processes, neutrophils generate large concentrations of reactive oxygen intermediates and decrease the levels of antioxidants, which are regulators of the apoptotic cascade [44]. IL-6 produced by MSCs dampens respiratory bursts from neutrophils but does not affect phagocytic activity, matrix adhesion, and chemotaxis [45]. The suppression of their releasing destructive enzymes, such as peroxidases and proteases, rescues neutrophils from apoptosis [45, 46].

2.5. Macrophages. PGE2 secreted by MSCs influences the macrophage switch from an inflammatory M1 into an anti-inflammatory M2 state [47–49]. This M2 macrophage expresses high levels of CD206 and IL-10, reduces levels of TNF-α and IL-12, and shows higher phagocytic activity [50, 51]. In addition, the shift in macrophage polarization was observed *in vitro* and *in vivo* using EVs isolated from human AT-MSCs [52]. Morrison's group demonstrated this in an acute respiratory distress syndrome murine model using human-derived MSCs and postulated an EV-mediated mitochondrial transfer [53].

2.6. Dendritic Cells (DCs). DCs, the most efficient antigen-presenting cells, prime naïve T cells to activate the adaptive immune cascade and interact with MSCs [54]. MSCs block the differentiation of monocytes towards DCs through a mechanism involving PGE2 [55] and prompt the

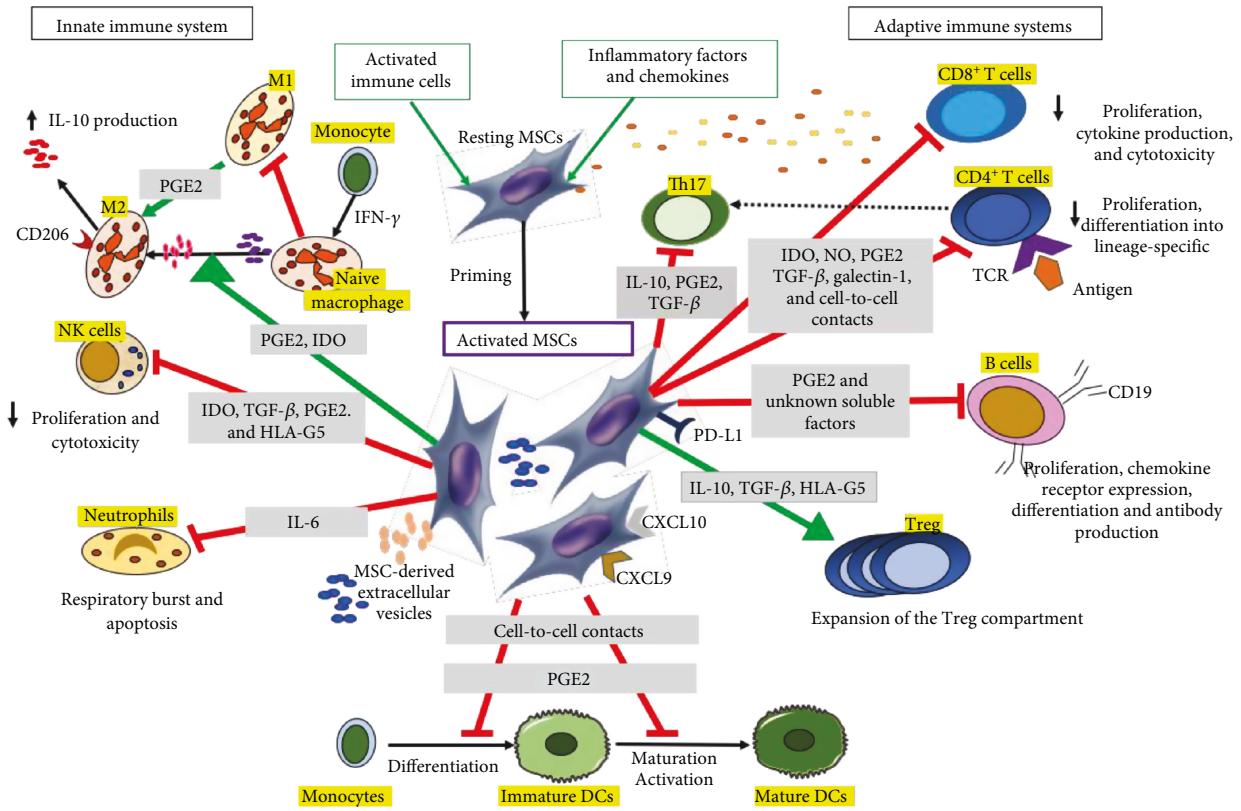

Figure 1: Mechanisms mediating immunomodulation. MSCs and their derived extracellular vesicles (EVs) exert their effect on innate (NK, neutrophils, monocytes, and macrophages) and adaptive (B and T cells) immune systems, as well as dendritic cells (DCs) through cell-to-cell interactions and several immunomodulatory factors. Activated T cells activate resting MSCs, which in turn facilitate the recruitment of helper and effector T cells via CXCL9 and CXCL10. Several immunomodulatory factors (TGF-β, PGE2, and HLA-G5) and membrane-bound molecules (PD-L1) suppress $CD4^+$ and $CD8^+$ T cell proliferation and induce the polarization of $CD4^+$ T cells towards Th17 cells. NO and IDO released by MSCs act on the suppression of $CD8^+$ T cell proliferation, cytokine production, and cytotoxicity. MSCs support the development of Treg populations via IL-10, TGF-β, and HLA-G5. In the context of B cells, MSCs inhibit activation, proliferation, chemokine receptor expression, and differentiation to antibody-secreting plasma cells. MSCs suppress naïve macrophage polarization to proinflammatory M1 macrophage and then favor anti-inflammatory M2 polarization. IL-6 secreted by MSCs suppresses neutrophil apoptosis and respiratory burst.

differentiation of mature DCs into a regulatory subtype through cell-cell contact, involving Jagged-2 [56].

Figure 1 summarizes some of the mechanisms mediating immunomodulation.

3. Value of Preconditioning MSCs

3.1. Preconditioning MSCs to Enhance Immunomodulation. MSCs do not inherently display immunosuppressive properties at baseline. To replicate the inflammatory environment of a patient suffering from immune dysfunction, they require activation to adopt an immunosuppressive phenotype [57, 58]. In addition to the inflammatory status of the recipient, the efficacy of MSC-based therapies is influenced by differences in tissue origin, donor-to-donor heterogeneity, and dearth of standardized manufacturing practices [19, 21]. Ongoing research efforts are focused on "licensing" or "priming" MSCs to display a more homogeneous immunosuppressive phenotype. This concept refers to an *in vitro* exposure of MSCs to proinflammatory cytokines such as IFN-γ, TNF-α, IL-1α, or IL-1β [14]. Other preconditioning cytokines and stimuli such as hypoxia and pharmacological agents can also be used during *in vitro* culture to modulate the MSC secretory profile [59] and thus impact their properties [60]. Preconditioning strategies also extend to methods of triggering the expression of cytoprotective genes that aim at prolonging the longevity of MSCs introduced to an adverse inflammatory milieu and therefore extend the duration of the immunomodulatory effect exerted [61]. These stimuli appear to potentially "correct" such variation and therefore allow the use of more uniform therapeutic products with enhanced immunosuppressive potential, which may lead to higher clinical benefits in patients. Although strategies for improving MSC function are advancing at the bench, there are other factors to be considered before their implementation in the clinic. Nowadays, the assessment of functionally relevant markers reflecting the immunoregulatory properties of MSCs should become the basis for their clinical use as therapeutic cell-based products. Scientists at the U.S. Food and Drug Administration (FDA) designed an assay that identifies morphological changes associated with the immunosuppressive capacity after priming. By integrating the analysis of cellular

changes with high-dimensional flow cytometry data and quantification of IFN-γ-augmented immunosuppression from multiple experimental conditions into a singular experiment, they were able to obtain a predictive measurement of the immunosuppressive capabilities of the cells [62].

3.2. Preclinical Studies Using Primed MSCs. Recent preclinical reports in the literature have demonstrated the significance of MSC priming with inflammatory cytokines for future clinical use. In addition to the aforementioned agents, others such as hyaluronan, polyinosinic acid, and polycytidylic acid have been used to prime MSCs for several forms of connective tissue repair in mice [63, 64]. These primed MSCs exhibit enhanced therapeutic properties with minimal or no significant adverse effects when compared to unprimed (naïve) counterparts [65, 66]. MSCs from multiple sources such as AT, BM, and Wharton's Jelly (WJ) primed with IFN-γ displayed gene expression profiles consistent with an immunosuppressive potential [67]. The immunomodulatory properties of MSCs derived from UC, AT, and periodontal ligaments presented comparable immunosuppressive capacities *in vitro*; however, UC-MSCs had shorter expansion time, predominantly utilized HLA-G as an immunosuppressive mechanism, and upon activation with IFN-γ did not express further HLA-DR, which would lower the risk of triggering an allogeneic immune response [68]. When IFN-γ-primed BM-MSCs isolated and cultured under good manufacturing practice (GMP) conditions were infused into murine models, no adverse effects related to primed BM-MSCs administration were found. Furthermore, the comparison of phenotypic profiles between primed and unprimed MSCs from the same donor demonstrated that the changes were due to IFN-γ priming rather than genetic variability [66]. In the context of graft versus host disease (GvHD), GvHD-mice injected with IFN-γ-primed MSCs had improved survival rates when compared to the group injected with naïve cells, and this was attributed to the activation of the IFN-γ-Janus kinase- (JAK-) signal transducer and activator of transcription 1 (STAT 1) pathway, which suppressed T cell proliferation [65].

4. Clinical Applications of MSCs in Diseases Mediated by Immune Cells

Culture-expanded MSCs are classified by both the FDA and European Medicines Agency (EMA) as more than minimally manipulated cellular and gene therapy (CGT) products [69]. The earliest therapeutic attempts at using autologous MSC infusion after ex vivo culture expansion showed an acceleration of the hematopoietic reconstitution after hematopoietic stem cell transplantation [70] and high-dose chemotherapy in breast cancer [71]. In both studies, no treatment-associated adverse effects were reported, thus these results laid the foundation for ex vivo cell expansion and administration. While the majority of MSC applications so far have relied on BM being the gold standard source, other adult and fetal tissues such as AT, UC, and WJ have gained popularity because of their comparable or even superior immunomodulatory profiles and their accessibility as medical waste products [72, 73]. For early phase human clinical trials, several factors including identity, viability, and sterility are established as release criteria [8]. However, for advanced-phase clinical trials, regulatory authorities additionally required the development of potency assays as part of the release criteria [74]. Additionally, the EMA has provided multiple guidelines to ensure quality, safety, and efficacy, including the "Guideline on Human Cell-Based Medicinal Products," "Guideline on Strategies to Identify and Mitigate Risk for First-in-Human Clinical Trials with Investigational Medicinal Products," and "Reflection Paper on Stem Cell-Based Medicinal Products," among others [75].

4.1. Broad Range of Applications. Most of the clinical trials performed to date have showed the feasibility and safety of the approach with however conflicting results in terms of efficacy, partially explicable with methodological biases (i.e., small cohorts, lack of control groups, variability of source, and preparation and routes of administration). Also, the use of autologous *vs.* allogeneic MSC is still controversial with no univocal data on the immunological properties of MSCs derived from patients suffering from autoimmune disorders compared to healthy donors [76, 77]. We provide a brief overview of clinical trials performed or ongoing in the setting of immune-related disorders. However, a more comprehensive picture is beyond the scope of the current review.

Results of clinical trials in inflammatory bowel disease have been recently reviewed by Algeri et al. [76]. MSCs have been administered intravenously to control luminal inflammatory disease or locally in perianal fistulizing Crohn's disease (CD), in cases of refractory disease or acute flares not responsive to conventional methods of treatment such as steroids and immunosuppressive drugs. The two largest studies conducted on systemic administration of allogeneic MSCs have reached conflicting conclusions: Lazebnik et al. showed clinical response in all treated patients (39 Ulcerative Colitis and 11 CD, [78]), while Pfizer did not succeed to demonstrate any clinical benefit in 48 treated Ulcerative Colitis patients compared to 40 placebo [79].

More homogenous positive results have been obtained for the treatment of fistulizing CD where MSCs promote the healing of rectal mucosa, without any observable adverse events [80–82]. A phase III randomized, double blind, controlled trial with allogeneic, adipose-derived MSCs (Cx601) demonstrated a higher remission rate in 107 patients treated *vs.* 105 placebo [81]. Alofisel or Cx601 is going to be the first off-the-shelf MSC therapy to be approved by EMA for complex perianal fistulas in adult CD [83].

Since 2004, allogeneic MSCs have been used in the treatment of GvHD in several patients enrolled in a multitude of trials worldwide [10, 84]. Osiris sponsored a phase III trial of allogeneic BM-MSCs from random donors for the treatment of steroid-refractory GvHD (NCT00366145). Unfortunately, it was considered a failure due to a lack of positive outcomes [85]. This was due to inconsistencies in sourcing, isolation and manufacturing methods, passage numbers used, and fresh *vs.* thawed cells [86, 87]. Despite this, the Osiris-backed BM-MSC product has been approved in Canada, New Zealand, and Japan (on an insurance-dependent

Table 1: Clinical trials of MSCs on diseases mediated by the immune system.

Trial no.	Phase	Commencement year	Targeted disease	Status	Patient enrollment (n)	Country
NCT00447460	I/II	2007	Graft vs. host disease (GvHD)	Completed [100]	15	Spain
NCT01522716	I	2011		Unknown (NRP)	11	Sweden
NCT01764100	I	2013		Completed [101]	40	Italy
NCT02032446	I/II			Recruiting	47 (estimated)	
NCT02291770	III	2014		Unknown (NRP)	130 (estimated)	China
NCT02055625	I/II			Suspended (NRP)	11	Sweden
NCT02359929	I	2015		Recruiting	24 (estimated)	USA
NCT01741857	I/II		Systemic lupus erythematosus (SLE)	Completed [102]	40	China
NCT03171194	I			Active, not recruiting	6 (estimated)	USA
NCT03673748	II	2019	SLE/lupus nephritis	Not yet recruiting	36 (estimated)	Spain
NCT00781872	I/II	2006	Multiple sclerosis (MS)	Completed [103]	20	Israel
NCT00395200	I/II	2008		Completed [104, 105]	10	UK
NCT01730547	I/II	2013		Unknown	15 (estimated)	Sweden
NCT02495766	I/II	2015		Unknown	8 (estimated)	Spain
NCT03799718	II	2019		Not yet recruiting	20 (estimated)	USA
NCT02893306	II	2012	Type 1 diabetes mellitus (T1DM)	Unknown (NRP)	10	Chile
NCT02940418	I	2017		Recruiting	20 (estimated)	Jordan
NCT03406585	I/II			Recruiting	24 (estimated)	Sweden
NCT02249676	II	2013	Devic syndrome/ neuromyelitis optica	Completed	15	China
NCT01659762	I	2012	Crohn's disease	Completed [106]	16	USA

NRP: no results posted.

basis) for restricted use in children with GvHD [88]. Alternative sources have also been tested, and placenta-derived decidua stromal cells seem to hold promise of better response rates compared to BM-MSCs for severe acute GvHD [89].

Rheumatic disorders are also considered another potential area for MSC application. Since 2010, more than 300 patients with relapsing systemic lupus erythematosus (SLE) have been reported in the same center in Nanjing, China. However, the presence of multiple biases in the study design (i.e., lack of endpoint definition and of randomization) and in data analyses renders the study inconclusive in proving efficacy. Regardless, the use of pooled allogeneic MSCs derived from healthy donors was also shown to regulate and normalize lymphocyte counts and differentials in SLE patients [90].

Similarly, phase I/II uncontrolled clinical trials have been conducted in other inflammatory rheumatic diseases, such as systemic sclerosis, Sjögren syndrome, dermatomyositis/polymyositis, and rheumatoid arthritis with promising results, although bigger randomized prospective controlled studies are mostly warranted [91, 92]. Several ongoing clinical trials are exploring the efficacy and toxic effects of MSCs in patients with multiple sclerosis [93]; however, phase I/II studies have not brought significant positive results and further investigations are warranted [94, 95]. In a large nonrandomized comparative trial in 173 patients with active rheumatoid arthritis, the intravenous treatment with UC-MSCs succeeded in inducing a substantial remission of the disease as per the American College of Rheumatology improving standards [96]. Based on the fact that several studies in animal models of Type 1 Diabetes (T1D) have shown MSCs to ameliorate or reverse overt diabetes, also demonstrating their successful engraftment in the pancreatic islets [97, 98], Carlsson et al. performed a phase I clinical trial showing for the first time the opportunity to interfere with the progression of T1D by systemic infusion of MSCs. Autologous BM-MSCs were administered to adult patients recently diagnosed with T1D. Strikingly, during the first year postdiagnosis, no adverse events were disclosed and a conserved or improved C-peptide response to a mixed-meal tolerance test in the patient cohort was demonstrated [99].

Table 1 summarizes other clinical trials of MSCs on diseases mediated by the immune system not previously discussed (http://www.clinicaltrials.gov, [100–106]).

4.2. Current Challenges in Clinical Use

4.2.1. Fate of the Infused MSCs. A factor that influences the future of MSC application in the clinic is that the exact fate of the cells postinfusion is yet to be completely elucidated. There are multiple reports in both human and animal models that point to sequestration of the cells in the lungs following systemic administration and their complete disappearance within 7 days of treatment [9, 107, 108].

Another study showed that allogeneic donor MSC DNA was found engrafted into the recipient's digestive tract *via* chromosomal fluorescence studies [10]. This is in support of the theory that MSCs are capable of escaping sequestration and migrating to sites of inflammation, homing to released cytokines and other inflammatory molecules. If this is the case, this will facilitate the administration of MSCs to patients with multisystemic or disseminated involvement, e.g., SLE and rheumatoid arthritis, with gross effects including treating inflammation, regulating lymphocyte function, and stimulating tissue repair, including regeneration of cartilage [109]. Other theories suggest that MSCs prior to apoptosis release EVs that are capable of migrating to inflamed tissues and exerting the same anti-inflammatory effects of viable MSCs. This alternative approach highlights the potential of cell-free MSC-based therapy [52, 107].

4.2.2. Practical Decisions Impacting MSC-Based Therapy Outcome. Other dilemmas impacting the widespread clinical use of MSCs that researchers have yet to reach a consensus for are which tissue source yields the most effective product, combined with the significant impact of donor variability and continued passaging on cell growth, protein production, and EV release [85, 110]. Furthermore, there is a lack of standardized disease-specific procedures and clinical trial regulations regarding the magnitude (average of 1-2 million cells/kg body weight) and frequency of dose administration, the use of allogeneic *vs*. autologous MSCs, systemic *vs*. local administration, and primed *vs*. naïve cells, and the use of freshly cultured *vs*. frozen and thawed cells [76]. Functional differences were observed between *in vivo* and *in vitro* contexts and between species (murine *vs*. human) in terms of susceptibility to undergoing oncogenic transformation during expansion, and effector molecules used in T cell suppression mechanisms have to be taken into account [21]. This is highlighted by the reported discrepancies between what is described in *in vitro* and animal models *vs*. what is reported in the literature of later-phase clinical trials and by the publishing bias (few or no reports on negative outcomes and/or failed trials) [92]. Interestingly, the lack of consistent benefit seen in late phase human clinical trials may also be explained by the fact that the injected cell products were "naïve or resting" MSCs; therefore, the immunosuppressive potential of the cells is entirely depending on an individual patient's microenvironment and immune status [19, 21, 111]. These variables collectively hinder the production of reliable "off-the-shelf" cell therapy products that produce sustainable and consistent results among patients.

5. Risk of Modulating the Action of Immune Cells and the Dilemma of Cancer Formation and Development

One of the main concerns in MSC-based therapy is that tumorigenicity could result from MSC malignant transformation during *in vitro* culture expansion or following infusion, or the immunosuppressive effects exerted by MSCs could allow tumor formation and development of already existing malignant cells in the host/recipient [112]. Similarly to murine MSCs readily undergoing spontaneous transformation *in vitro* [113], Rosland et al. demonstrated spontaneous malignant transformation of BM-derived human MSCs after *in vitro* cultures leading to an aggressively metastatic disease in immunodeficient mice [114]. However, the impaired immunological status of the recipient was likely more prone to initiate or develop cancer [115]. In humans, MSCs are minimally susceptible to oncogenic transformation *in vivo*, and long-term culture either does not affect MSC morphology or cause chromosomal alterations [116]. Furthermore, continued passaging leads to loss of already existing aneuploidy, or any resulting aneuploidy leads to senescence, negating the risk of cancer formation [117]. The Committee for Advanced Therapies and the Cell Product Working Party organized a meeting to discuss the risk of tumor formation following MSC-based therapies, with a focus on regulatory and scientific aspects. When discussing the influence of the manufacturing process on inducing cytogenetic abnormalities, it was highlighted that culture duration and conditions present critical risk factors for producing chromosomal aberrations. The committee also suggested that long-term expansion could mostly cause chromosomal aberrations rather than donor-derived factors [112]. However, in a study by Tarte et al., aneuploidy without risk of transformation occurring in a long-term culture of clinical grade MSCs was most likely donor dependent (3 out of 5 aberrations were derived from the same donor) [118]. Thus, donor screening and monitoring of the long-term expansion and integrity of the cells are a requirement [119].

MSCs exhibit a tropism for the tumor microenvironment niche [120], and selective homing into inflammatory tumor sites has been established in various types of cancer [121]. Even if MSCs have intrinsic antitumor properties, they can potentially alter their phenotype towards a protumorigenic role including proangiogenic and immunosuppressive capabilities. Thus, the presence of MSCs within the cancerous stroma has been a matter of contradictory reports [122]. There is no official statement on the potential of tumorigenicity in MSC-based therapies, and no observation of tumor formation of MSC origin in patients given cellular therapy. Despite these facts, one cannot rule out the possibility of MSC-derived tumors developing *in vivo*. Interestingly, there are reports of spontaneous MSC transformation resulting from MSC culture cross-contamination with malignant cells emphasizing the importance of maintaining good manufactory practice conditions in the production of cell therapy products [123, 124]. While MSC therapy has been qualified as safe by both FDA and EMA, the potential long-term risks still have to be considered.

6. Conclusion

In the last 10 years, MSCs have been a promising treatment for a plethora of immune-related conditions, through the regulation of inflammation and the support of tissue homeostasis. Despite having been unanimously deemed safe, clinical trials report conflicting data in terms of efficacy in several clinical settings. Inconsistencies can be ascribed to

limitations in the design of clinical trials and translation of successful preclinical models, discrepancies in the source, preparation and handling of the MSC product, route of administration, and type of donor (autologous *vs.* allogeneic).

Moreover, the lack of *in vitro* biomarkers correlating with the *in vivo* activity of MSCs has so far hindered the progress towards uniformly potent cell products. MSC priming or licensing, before administration, might offer the possibility to enhance their effectiveness *in vivo*, limiting the variability inherent to the inflammatory status of the patients.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

The Immunomodulatory Potential of Wharton's Jelly Mesenchymal Stem/Stromal Cells

Fernanda Vieira Paladino,[1,2] **Juliana de Moraes Rodrigues**,[1] **Aline da Silva**,[1] **and Anna Carla Goldberg**[1,2,3]

[1]*Hospital Israelita Albert Einstein, São Paulo, Brazil*
[2]*Departamento de Alergia e Imunopatologia, Faculdade de Medicina, Universidade de São Paulo, Brazil*
[3]*Instituto de Investigação em Imunologia-INCT, Brazil*

Correspondence should be addressed to Anna Carla Goldberg; goldberg@einstein.br

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The benefits attributed to mesenchymal stem/stromal cells (MSC) in cell therapy applications are mainly attributed to the secretion of factors, which exhibit immunomodulatory and anti-inflammatory effects and stimulate angiogenesis. Despite the desirable features such as high proliferation levels, multipotency, and immune response regulation, there are important variables that must be considered. Although presenting similar morphological aspects, MSC collected from different tissues can form heterogeneous cellular populations and, therefore, manifest functional differences. Thus, the source of MSC should be a factor to be considered in the development of novel therapies. The following text presents an updated review of recent research outcomes related to Wharton's jelly mesenchymal stem/stromal cells (WJ-MSC), harvested from umbilical cords and considered novel and potential candidates for the development of cell-based approaches. This text highlights information on how WJ-MSC affect immune responses in comparison with other sources of MSC.

1. Introduction

Mesenchymal stem/stromal cells (MSC) are increasingly viewed as sources of cell therapy applications due to their known immunomodulatory and anti-inflammatory effects and capacity to stimulate angiogenesis. Despite the desirable features such as high proliferation levels, multipotency, and immune response regulation, there are important variables that must be considered. Although presenting similar morphological aspects, MSC collected from different tissues can form heterogeneous cellular populations and also manifest tissue-specific functional differences. Thus, the source of MSC should be a factor to be considered in the development of novel therapies. The following text presents an updated review of recent research outcomes related to Wharton's jelly mesenchymal stem/stromal cells (WJ-MSC), harvested from umbilical cords and considered novel and potential candidates for the development of cell-based therapies. This text highlights information on how WJ-MSC affect immune responses in comparison with other sources of MSC. Some of the challenges to be addressed in order to overcome hurdles associated with the therapeutic application of these cells are also included.

2. The Umbilical Cord Is the Source of Wharton's Jelly

Wharton's jelly (WJ) can be generally described as the mucoid connective tissue that encloses the three umbilical vessels, one vein and 2 arteries, being surrounded by a single layer of amniotic epithelial cells, which constitute the human umbilical cord [1]. Recently, the ongoing interest in umbilical cords as a useful source of MSC encouraged further investigation on these tissue structures. WJ is currently divided into three main zones based on their histological appearance: (a) the subamnion with a sparse population of fibroblast-like

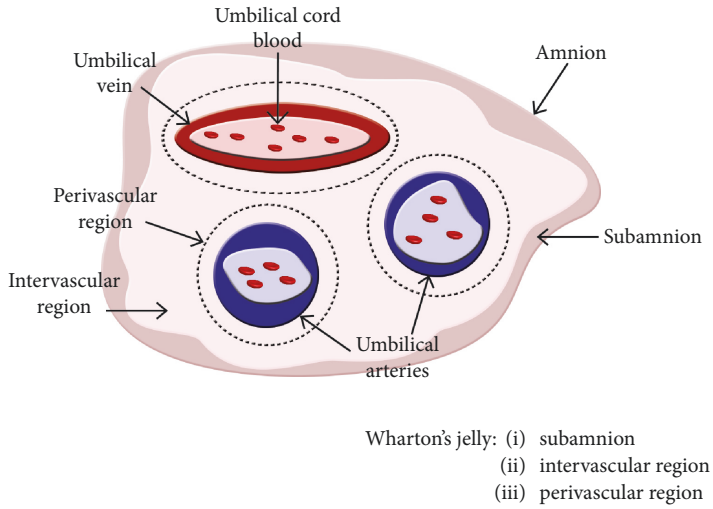

Figure 1: Human umbilical cord structure. Schematic image showing umbilical cord anatomical compartments, including Wharton's jelly.

cells; (b) the intervascular region, a matrix of connective tissue predominantly made from collagen I, which concentrates the greatest proportion of WJ-MSC; and (c) the perivascular layer that surrounds the umbilical vessels (Figure 1) [2, 3]. WJ-MSC derived from different parts of the same umbilical cord are equally valuable sources for use in cell therapy [4]. Of note, WJ-MSC are different from the hematopoietic stem cells found in the umbilical cord blood [5]. Moreover, as other authors already described [3], WJ is seeded by distinct sources of mesenchymal/stromal cells during the embryological development. These cell subsets express not only relevant markers that characterize both WJ-MSC and perivascular cells but possibly also the main source of progenitor cells that populate the WJ [6].

3. Benefits of Using WJ-MSC

MSC are considered a potential tool for cell therapy. The "gold standard" bone marrow-derived MSC (BM-MSC) are the most used in clinical trials but have shown mixed results [7–12]. Furthermore, their use is not always recommended due to the techniques needed to obtain the cell. BM-MSC are isolated from bone marrow aspirate; this is an invasive procedure and painful for the patient and is accompanied by a risk of infection, possibility of donor morbidity, differences in donor age, and still change or loss of *in vitro* proliferative and differentiation cellular capacity [13, 14].

Alternative sources where isolation is easier, like adipose tissue (AT) and WJ [15], should be and are being considered. AT is an autologous source of cells though some concerns like donor age and risk of infection are the same when compared to bone marrow (BM) [16]. Other alternative sources are, for example, dental pulp [17] and menstrual blood (reviewed in [18]), a well-recognized source of MSC known since 2004. The umbilical cord is usually discarded, mitigating the risks associated with the invasive procedures needed to isolate MSC from BM [16]. With few ethical concerns, WJ is considered an easily accessible source of MSC. WJ-MSC have been compared not only with BM-MSC but also with AT-MSC (adipose tissue-MSC) and MSC derived from menstrual blood [19] and in most cases, show higher proliferative capacity. In addition, WJ-MSC are very young cells derived from a protected neonatal tissue that has suffered less environmental interference, namely, the effects on the tissues resulting from disease history and life style, a fact that helps the acquisition of a more uniform cell cohort, which may favor their therapeutic application. However, the outcome of functional tests *in vitro* indicates that they too exhibit limited lifespans and variable immune suppression potentials [20–23]. WJ-MSC are less prone to develop defective functions that can accumulate throughout a cells' lifespan due to aging and the lifetime exposure to environmental factors [24]. It is important to take into account that quality control for these cells should follow specific criteria such as selecting samples from healthy donors of full-term pregnancies, women over 18 years of age, water broken for no longer than 18h, and the expectant mother must have had at least two consultations during pregnancy and should not present fever or infection at time of birth. Maternal serum screening before delivery should include hemoglobin electrophoresis and serology for prevalent viruses and parasites.

Several reports describe MSC as immune privileged or hypoimmunogenic cells, a status likely enhanced by immunologically protected neighbouring sites, the placenta and the foetus itself [25]. In fact, they express low levels of MHC class I and costimulatory CD40, CD80, and CD86. They also lack expression of MHC class II molecules [24, 26, 27], in spite of the observation of an upregulated HLA-DR expression on BM-MSC after treatment with IFN-γ, but not with TNF-α. Nevertheless, differing from BM-MSC HLA-DR expression, the same authors did not detect the effect on WJ-MSC [28]. WJ-MSC exhibit enhanced expression of immune suppression proteins, notably leukocyte antigen G6 (HLA-G6) known to have an important role in avoiding immune-based responses against the embryo, indoleamine-2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2) [29].

An important point for consideration is the fact that therapeutic applications involving MSC require an initial *in vitro* expansion step prior to their use and generally hundreds of millions of cells are used per treatment. It has been shown that several passages *in vitro* leads to a decrease in BM-MSC self-renewal capacity measured by telomere length shortening and increase in senescence markers [30]. Studies usually evaluate the immunomodulatory capacity of MSC from different sources only in early passages, and few data in the current literature is available on their behavior after passaging *in vitro* until enough numbers of cells are obtained for use in cell therapy [31, 32]. One study comparing AD-MSC and BM-MSC from passage 4 to passage 10 showed that they had similar cell morphology, surface marker expression, and immunomodulatory properties, even though gene expression was different [33]. Despite a higher lifetime *in vitro*, renewal of WJ-MSC ultimately will also lead to cell arrest and replicative senescence and the result will be the loss of stem cell functionality, even though the senescent cells remain alive [34–36]. We previously observed [20] that WJ-MSC from different donors exhibited different lifespans, as measured by senescent phenotype, number of passages, and expansion potential. Moreover, each WJ-MSC sample presented a unique behavior, differing in patterns of cytokine mRNA expression and immunomodulatory properties [37]. Thus, we believe that careful evaluation of senescence markers after repeated passaging plus monitoring of the immunosuppressant potential of each harvested cell must be included in quality control before therapeutic use.

4. Therapeutic Uses Based on the Immunomodulatory Effects of MSC: Comparing WJ-MSC with BM-MSC

When a tissue is damaged, inflammation occurs and tissue-resident MSC and even BM-MSC are mobilized to the lesion site [29, 38]. Because of their multipotency, it was believed that recruited MSC differentiated into functional cells to replace the damaged ones. However, this occurrence has eluded researchers. Studies using autologous cells mainly from bone marrow and adipose tissue and/or allogeneic cells from umbilical cord blood have shown that after infusion transdifferentiation of MSC into functional cells in tissues rarely occurs if at all [39]. In turn, it has become increasingly clear that in response to an inflammatory milieu, MSC prepare the microenvironment for tissue repair by producing immunoregulatory molecules that modulate the progression of inflammation, releasing growth factors to produce extracellular matrix [40], stimulating the *in situ* progenitor cells to differentiate and replace lost cells [41], and promoting angiogenesis [42]. The apparent incongruity between the benefit achieved and the lack of differentiation of the recruited MSC into specialized tissue cells has led to the unraveling of the surprising immunosuppressive capacity of MSC from many different sources [43–48].

By now, it is well known that the most promising benefits of therapy with MSC occur in patients presenting inflammatory or autoimmune diseases [49, 50]. Thus, the MSC immunomodulatory effects may play an important role in the improvement of autoimmune diseases like systemic lupus erythematosus [51, 52], type 1 diabetes mellitus [53], and multiple sclerosis [54, 55]. Ringden and Le Blanc showed that treatment using an allogenic source of MSC from umbilical cord blood (UCB-MSC), not WJ-MSC, was able to reverse partially or totally GVHD in 50% of patients [56]. In addition, the group headed by Krampera et al. [57] and other researchers [45, 58, 59] sought to unveil the immunomodulatory mechanisms of BM-MSC, confirming their effect on proliferation and antigen-specific responses by T lymphocytes.

WJ-MSC also appear to show a robust immunomodulatory potential [22]. A comparative study using MSC derived from whole human umbilical cord (MC-MSC) WJ-MSC and BM-MSC showed that MC-MSC proliferated faster and survive longer in culture than WJ-MSC; however, they have similar immunomodulatory potential [60]. Another study comparing BM-MSC and WJ-MSC demonstrated that inflammation affects the immune properties of MSC sources in different ways. Priming BM-MSC enhanced the suppression of phytohemagglutinin (PHA) mitogen-stimulated T cells only, whereas IFN-γ-primed WJ-MSC were better suppressors of MLR (mixed lymphocyte reaction) [28]. BM-MSC, WJ-MSC, and AT-MSC were all capable of suppressing T cell proliferation [61, 62]. However, high levels of IL-17A were detected in WJ-MSC cocultures, which is one of the key mediators in the treatment of graft-versus-host disease [61]. In a murine experimental autoimmune encephalomyelitis (EAE) model, WJ-MSC treated with IFN-γ increased regulatory T (Treg) cell proliferation and decreased the secretion of inflammatory cytokines in EAE mice, reducing the symptoms of the disease [63].

Of note, human fetal bone marrow (FBM-MSC) and WJ-MSC have biological advantages as compared to adult cells [62]. WJ-MSC have a gene expression pattern similar to AT-MSC but not FBM-MSC. Beyond that, genes associated with cell adhesion, proliferation, and immunomodulatory function are increased in WJ-MSC as revealed by gene ontology. WJ-MSC intrinsically overexpress genes involved in neurotrophic support when compared to BM-MSC, which makes WJ-MSC an interesting candidate for cell therapy in neurodegenerative disorders [64].

5. MSC Exert Comprehensive Effects on Cell-Mediated Immune Responses

MSC can interact with and regulate the activation and function of immune cells, such as T and B lymphocytes [65, 66], dendritic cells (DC) [67], and monocytes/macrophages [68]. The effects of MSC on the immune system are generally anti-inflammatory and are achieved by different, but complementary mechanisms.

Nicola et al. showed that human BM-MSC are capable of suppressing T cell proliferation in a mixed lymphocyte reaction (MLR) or when T cells are activated by phytohemagglutinin (PHA) [45]. WJ-MSC suppress mitogen-induced T cell responses to a greater extent than either BM-MSC or AT-MSC [28]. Recently, our group showed that different samples

of human WJ-MSC were capable of inhibiting mitogen-activated CD3+ T cell proliferation, although to different extents, though the immunomodulatory profile of each WJ-MSC was essentially maintained even after 10 passages [37]. Another mechanism involved in immune suppression is T cell anergy. BM-MSC can induce T cell anergy by suppressing cyclin D2 expression and inhibiting CD4+ and CD8+ T cell proliferation by producing nitric oxide [69, 70]. BM-MSC are also capable of regulating the immune response by the induction of Treg, and it has been reported that they can induce T cell apoptosis via the Fas/FasL pathway. The apoptotic cells will stimulate macrophages to secrete high levels of TGF-β, which in turn will generate Treg cells [71]. Our preliminary results (unpublished data) showed that WJ-MSC were also able to induce Treg cells when cocultured with PBMC and treated with IFN-γ. BM-MSC also affect B cell functions, inhibiting the proliferation of activated B cells, their antibody production, and their chemotactic behavior [72]. BM-MSC have been shown to interfere in differentiation, maturation, and function of DC [67]. For example, in coculture, DC lose their ability to induce T cell activation [73–75]. Likewise, the differentiation of monocytes into mature DCs was inhibited and costimulatory ligand expression was blocked when cultured with WJ-MSC [76]. Taken together, the available literature indicates that WJ-MSC possess immunological features comparable to the better studied BM-MSC and even to MSC from other sources, but further detailing is needed to find the best therapeutic indications for this allogeneic source of cells as a substitute for the autologous BM-MSC and AT-MSC. The fact that WJ-MSC constitute an allogeneic therapy may in fact favor these cells in certain pathologies where the immunosuppressive response is urgent and should encompass cell and humoral responses.

An additional twist in this rationale is the observation that MSC, both *in vitro* and *in vivo*, seem capable of adopting a pro- or anti-inflammatory phenotype. Similar to the phenotype-switching phenomenon in macrophages massively explored throughout the literature and reviewed elsewhere [77, 78], MSC are also sensitive to shifts in the local immune milieu. Disruption towards an excessive concentration of proinflammatory cytokines such as IFN-γ and TNF-α activates signalling pathways by way of sensors present on human BM-MSC, causing a shift to the MSC2 phenotype and playing an important role in the downregulation of immune cells and their corresponding proinflammatory mediators [79]. In contrast, to switch to a MSC1-type profile, an anti-inflammatory microenvironment is required and MSC1 will not only express lower levels of immunosuppressive genes including IDO, NO, and PGE2 but will also be a major source of proinflammatory molecules, which will recruit and activate immune cells by secreting IL-6 and producing IL-1α and IL-1β [78]. BM-MSC, under conditions of hypoxia and stimulated with proinflammatory cytokines such as IFN-γ, TNF-α, and IL-1β, increase the expression of Toll-like receptors TLR2, TLR3, and TLR4, rendering these cells more sensitive to the inflammatory medium [80]. Waterman et al. showed that BM-MSC acquired two distinct phenotypes after stimulation with TLR3 and TLR4 ligands and accordingly, resulted in different immunomodulatory effects. Indeed, LPS-stimulated BM-MSC (TLR4 ligand) exhibited a proinflammatory profile (MSC1) in contrast with the polyI:C stimulated BM-MSC (TLR3 ligand) that showed an anti-inflammatory profile (MSC2) [79]. The same group also showed that BM-MSC induced into expressing the MSC1 profile attenuate cancer cell growth while when the same cells exhibit a MSC2 phenotype, they act similarly to conventional MSC in promoting tumor growth and metastases [81].

The bottom-line result of MSC switching to a type 1 profile is ultimately an overall immune modulation opposing the local environment [78]. In an inflammatory milieu, the induction of a type 2 MSC will lead to the regulation of excessive immune responses at the focal point of injury, the desirable scenario to heal damaged tissue, sponsored and facilitated by MSC plasticity.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

IL-10 Gene-Modified Human Amniotic Mesenchymal Stem Cells Augment Regenerative Wound Healing by Multiple Synergistic Effects

Shune Xiao, Guangtao Huang, Zairong Wei, Kaiyu Nie, Zhiyuan Liu, Chengliang Deng, and Dali Wang

Department of Plastic Surgery, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou, China

Correspondence should be addressed to Chengliang Deng; cheliadeng@sina.com and Dali Wang; daliwangzy@sina.com

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Mesenchymal stem cells (MSCs) possess a capacity to enhance cutaneous wound healing that is well characterized. However, the therapeutic effect of MSCs appears to be limited. Modifying MSC target genes to increase necessary biological effects is a promising strategy for wound therapy. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that has a therapeutic effect on wound healing. In this study, we modified human amniotic mesenchymal stem cells (hAMSCs) using recombinant lentiviral vectors for expressing IL-10 and evaluated the therapeutic effects of hAMSCs-IL-10 in wound healing. We elucidated the mechanisms underlying the effects. We found that promoting wound healing was maintained by synergistic effects of hAMSCs and IL-10. hAMSCs-IL-10 showed stronger biological effects in accelerating wound closure, enhancing angiogenesis, modulating inflammation, and regulating extracellular matrix remodeling than hAMSCs. hAMSCs-IL-10 would be better at promoting wound healing and improving healing quality. These data may provide a theoretical foundation for clinical administration of hAMSCs-IL-10 in cutaneous wound healing and skin regeneration.

1. Introduction

Wound healing is a complex process that includes inflammation, cell proliferation, angiogenesis, and extracellular matrix (ECM) remodeling [1]. Since scar-free regenerative wound healing of human fetuses was reported, efforts have been directed at investigating the underlying mechanisms by comparing the wound-healing processes of scarless and scarring wounds in multiple animal models. A key difference identified in fetal wound healing is a low inflammatory reaction compared to postnatal wounds. Interleukin- (IL-) 10 is essential for the ability of fetal wounds to have low inflammatory reactions for scarless regenerative wound healing [1, 2]. Misalignment of biodynamic processes can lead to delayed healing and excessive scarring, which present large challenges to healthcare systems globally.

Mesenchymal stem cells (MSCs) are widely reported to have an active function in the process of wound healing [3]. MSC-based skin engineering combined with genetic recombination in which MSCs are the seed cells and the vehicle for gene delivery to the wound site represents the most promising option for a strategy for wound therapy [4]. Alapure et al. found that bone marrow MSCs with incorporated biomaterial covering burn wounds promote closure, reepithelialization, granulation tissue formation, and vascularization of burn wounds [5]. Modification of MSCs by hepatocyte growth factor and vascular endothelial growth factor (VEGF) genes to increase necessary biological effects and augment wound healing has been confirmed [6, 7].

IL-10 is an anti-inflammatory and antifibrotic cytokine. It is essential for the ability of a fetus to heal regeneratively [1, 2]. IL-10 has been shown to recapitulate scarless regenerative healing in postnatal tissue through pleiotropic effects. Besides regulating the inflammatory response, IL-10 has novel functions as a regulator of the extracellular matrix, fibroblast cellular function, and endothelial progenitor cells [8–10]. Given this information, we hypothesized that

overexpression of IL-10 in MSCs may have beneficial effects on MSCs facilitating regenerative wound healing and preventing scar formation. In this study, we evaluated the therapeutic effects of IL-10 gene-modified hAMSCs (hAMSCs-IL-10) on anti-inflammation and antifibrosis effects and promotion of wound healing.

2. Materials and Methods

2.1. Animals and Ethics Approval. Wild-type, 7- to 8-week-old C57BL/6 mice were provided by the Animal Experimental Center of the Army Military Medical University (Chongqing, China). Human placentas were obtained from donors following normal or cesarean deliveries after obtaining informed consent and approval from the Affiliated Hospital of Zunyi Medical University Institutional Review Board. All experimental procedures were performed in accordance with the guidelines and regulations established by the Medical Ethics Committee of Zunyi Medical University (Zunyi, China).

2.2. Isolation, Culture, and Flow Cytometry Identification of hAMSCs. hAMSCs were isolated and cultured as previously described, with slight modifications [11]. The amnion was separated from the chorion mechanically and rinsed three times in phosphate-buffered saline (PBS) with 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA). The amnion was cut into small pieces and incubated with 0.25% trypsin/EDTA (0.05%, Gibco) at 37°C for 40 min to remove amniotic epithelial cells. After rinsing with PBS, amnion fragments were minced and digested with 0.75 mg/mL collagenase II (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 90 min with gentle shaking. An equal volume of Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) was added to stop the enzymatic reaction, and cell suspensions were filtered with 100 μm mesh cell strainers. Cell suspensions were centrifuged at 500 $\times g$ for 5 min, and cell pellets were resuspended and cultured in DMEM/F12 medium (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin. At 80% confluence, hAMSCs were subcultured, and cells at passage 3 were used in following experiments.

Flow cytometry was used to identify characteristics of hAMSCs and detect stem cell-related cell surface markers. For flow cytometry, 1×10^6 cells/100 μL were collected and stained with antibodies against CD90 (FITC, clone: 5E10), CD105 (PerCP-Cy5.5, clone: 266), CD73 (APC, clone: AD2), CD44 (PE, clone: G44-26), CD34 (PE, clone: 581), CD11b (PE, clone: ICRF44), CD19 (PE, clone: HIB19), CD45 (PE, clone: HI30), HLA-DR (PE, clone: G46-6), mIgG1 (κ FITC, clone: X40), mIgG1 (κ PerCP-Cy5.5, clone: X40), mIgG1 (κ APC, clone: X40), mIgG1 (κ PE, clone: X40), mIgG2a (κ PE, clone: G155-178), and mIgG2b (κ clone: 27-35). All antibodies were used at 1 : 1000 (BD, Pharmingen, USA). Samples were incubated at room temperature for 30 minutes, washed with PBS, and analyzed with a MoFlo XDP flow cytometer (Beckman Coulter, Brea, CA) with Kaluza software (Beckman Coulter).

2.3. Osteogenesis and Adipogenesis of hAMSCs. Multilineage differentiation of hAMSCs was tested. To induce differentiation into osteocytes and adipocytes, cells were cultured in osteocyte differentiation medium or adipocyte differentiation medium. After 14 days of differentiation, cells were stained with Alizarin Red S (Cyagen, Guangzhou, China) or Oil Red O (Cyagen).

2.4. Construction and Characterization of IL-10-Modified hAMSCs. An IL-10-overexpressing vector (carrying green fluorescent protein) was from Shanghai Innovation Biotechnology Co. Ltd. The multiplicity of infection (MOI) was 30. Lentiviruses used in this study were LV-IL-10, a replication-defective lentivirus expressing IL-10, and LV-Null, a replication-defective lentivirus not carrying any exogenous genes. The hAMSCs were transfected with 30 MOI of LV-IL-10 (hAMSCs-IL-10) or LV-Null (hAMSCs-Null) and observed under a fluorescence microscope at 48 h postinfection. Expression of IL-10 in supernatants of hAMSCs-IL-10, hAMSCs-Null, and hAMSCs was detected by the enzyme-linked immunosorbent assay (ELISA).

2.5. In Vivo Animal Studies. A full-thickness skin defect model was generated. C57BL/6 mice were anaesthetized by intraperitoneal injection of 10 g/L pentobarbital sodium (0.4 mL/100 g), and the dorsa were shaved, depilated, and cleaned with betadine. Two full-thickness excisional wounds (1 cm^2) were made symmetrically on both sides of the midline of the back. Mice were randomized into four groups and injected subcutaneously around the wound area with saline as a control (100 μL, $n = 10$), IL-10-hAMSCs (1×10^6 cells/100 μL saline, $n = 10$), hAMSCs-Null (1×10^6 cells/100 μL saline, $n = 10$), or hAMSCs (1×10^6 cells/100 μL saline, $n = 10$). Wound healing was evaluated on the basis of gross observation at days 1, 3, 7, and 14 after treatment, and the wound healing rate was calculated.

2.6. Inflammatory Response. To evaluate the anti-inflammatory effect of IL-10-hAMSCs, skin around wounds was collected at 3, 7, and 14 d after treatment, and inflammatory cell infiltration was observed by hematoxylin and eosin (H&E) staining. Expression levels of inflammatory factors IL-10, tumor necrosis factor-α (TNF-α), and IL-6 were quantitatively assayed with an ELISA assay kit (Sigma-Aldrich) following the manufacturer's instructions. The real-time fluorescence quantification polymerase chain reaction (qPCR) was used to detect relative mRNA levels for IL-10, TNF-α, and IL-6. For qPCR, total RNA was extracted from tissue around wounds using the RNAiso Plus reagent (Takara, Dalian, China); cDNA was synthesized from 2 μg total RNA with SYBR PrimeScript RT-PCR Kits (Takara), and qPCR was carried out using SYBR PrimeScript RT-PCR Kits on a Stratagene MX3005P qPCR system (Agilent Technologies, Santa Clara, CA, USA). All steps were performed according to the manufacturer's protocol. Fold change of each target gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA.

2.7. Evaluation of Vascularization. To observe angiogenesis during wound healing, skin around wounds was collected,

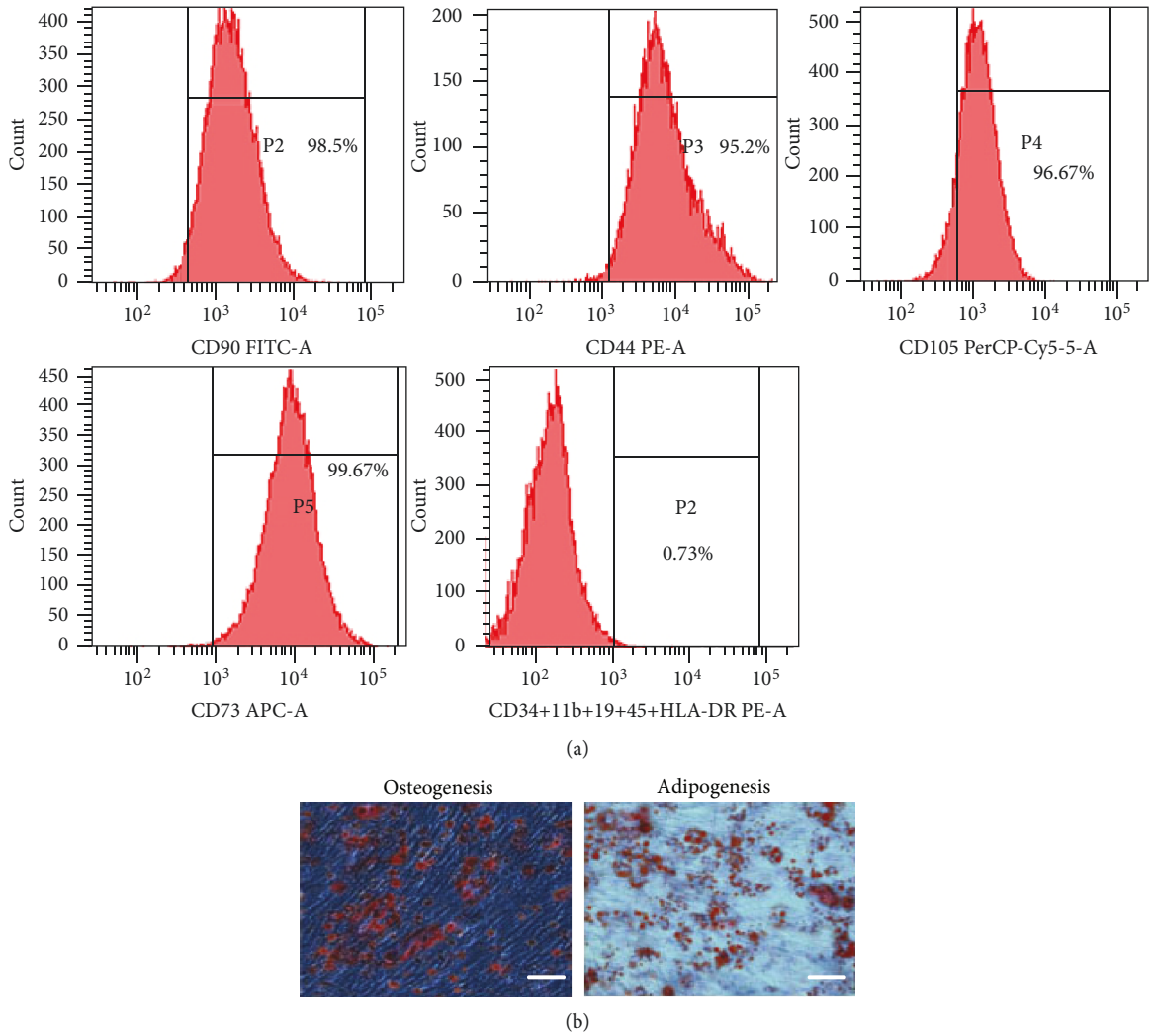

Figure 1: Characterization of hAMSCs. (a) FACS analysis of cell markers of hAMSCs. hAMSCs were >90% positive for CD105, CD73, CD44, and CD90 and negative for CD34, CD11b, CD19, CD45, and HLA-DR. (b) Differentiation of hAMSCs into osteocytes and adipocytes. Cells cultured under osteogenic or adipogenic culture conditions were stained for calcium deposits with Alizarin red staining or lipid droplets with Oil Red O staining. Scale bar = 50 μm. hAMSCs: human amniotic mesenchymal stem cells.

and microvessels were assayed by tissue H&E staining. Expression of angiogenic factors, VEGF, and basic fibroblast growth factor (bFGF) was detected with ELISA. For ELISA, the concentrations of VEGF and bFGF were quantitatively measured with a Quantikine enzyme-linked immunosorbent assay kit (Sigma-Aldrich) following the manufacturer's instructions.

2.8. Evaluation of Extracellular Matrix Remodeling. To explore the effect of IL-10-hAMSCs on ECM production and remodeling during the wound healing process, wound skin was collected, and collagen was assayed by Masson trichrome staining according to the manufacturer's instruction. Collagen content was calculated by ImageJ software as the percentage of stained area to total area of the section. Expression of transforming growth factor-β (TGF-β), matrix metalloprotein-1 (MMP-1), and tissue inhibitor of metalloprotein-1 (TIMP-1) was measured with ELISA and immunohistochemical staining. For immunohistochemical staining, antibodies were against TGF-β (1 : 150, Abcam), MMP-1 (1 : 200, Abcam), and TIMP-1 (1 : 250, Abcam).

2.9. In Vivo Tracing. To follow the fate of hAMSCs *in vivo*, untransfected hAMSCs were labeled with a cell tracker CM-DiI (Invitrogen, Grand Island, NY, USA) prior to grafting, according to the manufacturer's instructions. hAMSCs-IL-10 and hAMSCs-Null expressed green fluorescence protein. On day 14, wound skin was harvested, and frozen sections were prepared. Survival of cells was observed under a fluorescent microscopic system (IX71 FL, Olympus, Japan).

2.10. Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). One-way analysis of variance was performed for comparison between different groups using SPSS 17.0 software (IBM, Armonk, NY, USA). Differences with $P < 0.05$ were regarded as statistically significant.

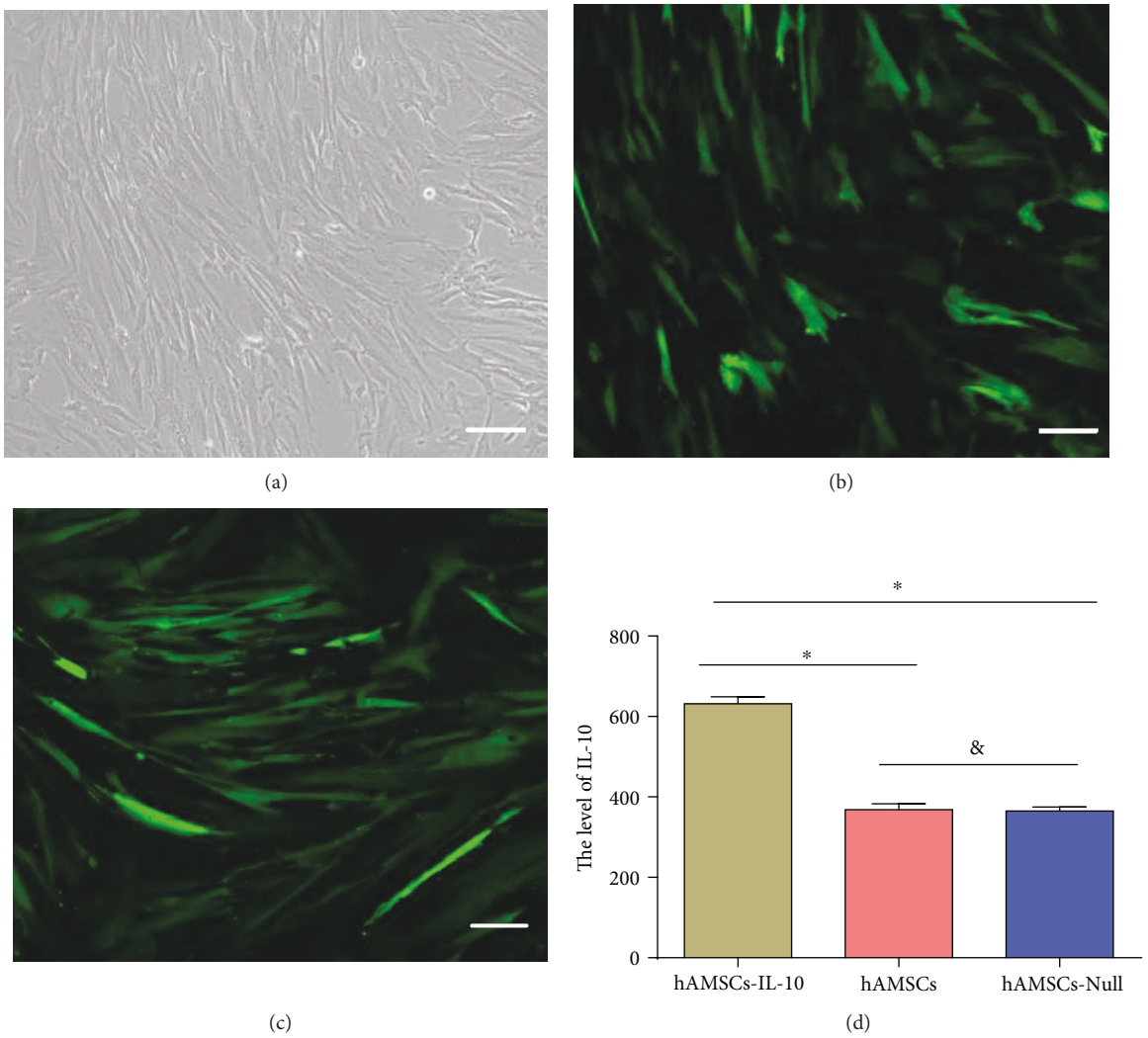

Figure 2: Morphology, transfection efficiency, and IL-10 expression of hAMSCs. (a) Phase-contrast micrograph of hAMSCs showing spindle-shaped morphology. (b) Fluorescence micrographs of hAMSCs after LV-IL-10 infection for 48 h. (c) Fluorescence micrographs of hAMSCs after LV-Null infection for 48 h. (d) IL-10 expression of hAMSCs; the concentration of IL-10 from hAMSCs-IL-10 increased compared to that of hAMSCs-Null and hAMSCs. $^{*}P < 0.05$ and $^{\&}P > 0.05$. LV-IL-10: replication-defective lentivirus expressing IL-10; LV-Null: replication-defective lentivirus not carrying any exogenous genes. Scale bar = 500 μm.

3. Results

3.1. Characterization of hAMSCs. Flow cytometry showed expression of surface markers of hAMSCs. hAMSCs were >90% positive for CD105, CD73, CD44, and CD90 and negative for CD34, CD11b, CD19, and CD45 (Figure 1(a)). In addition, hAMSCs were negative for HLA-DR, indicating that they possessed low immunogenicity (Figure 1(a)). hAMSCs differentiated into osteocytes as demonstrated by positive Alizarin Red staining and adipocytes as shown by Oil Red O staining (Figure 1(b)). These results indicated that cultured hAMSCs possess stem cell characteristics.

3.2. Transfection Efficiency and IL-10 Expression of hAMSCs. Cultured hAMSCs were spindle-shaped with a relatively high nucleus-to-cytoplasm ratio (Figure 2(a)). After infection with lentivirus (LV-IL-10 and LV-Null), hAMSCs were observed under a fluorescence microscope at 48 h postinfection. The cells showed a uniform spindle shape and a high transfection rate of 90% (Figures 2(b) and 2(c)). We detected levels of IL-10 in the supernatant by ELISA at 48 h after infection of hAMSCs. Expression of IL-10 from hAMSCs-IL-10 increased compared to hAMSCs-Null and hAMSCs ($P < 0.05$) (Figure 2(d)).

3.3. IL-10 Promoted Wound Recovery. The complete healing of cutaneous wounds with good epithelization was evaluated by general observation. Wound healing in the hAMSCs-IL-10 group was more rapid than in the other three groups. At days 3 and 7 after cell transplantation, the hAMSCs-IL-10, hAMSCs-Null, and hAMSC groups showed significantly higher wound healing than the control. The hAMSCs-IL-10 group had significantly smaller wound sizes than the other groups between days 3 and 7. On day 14, the hAMSCs-IL-10, hAMSCs-Null, and hAMSC groups achieved complete

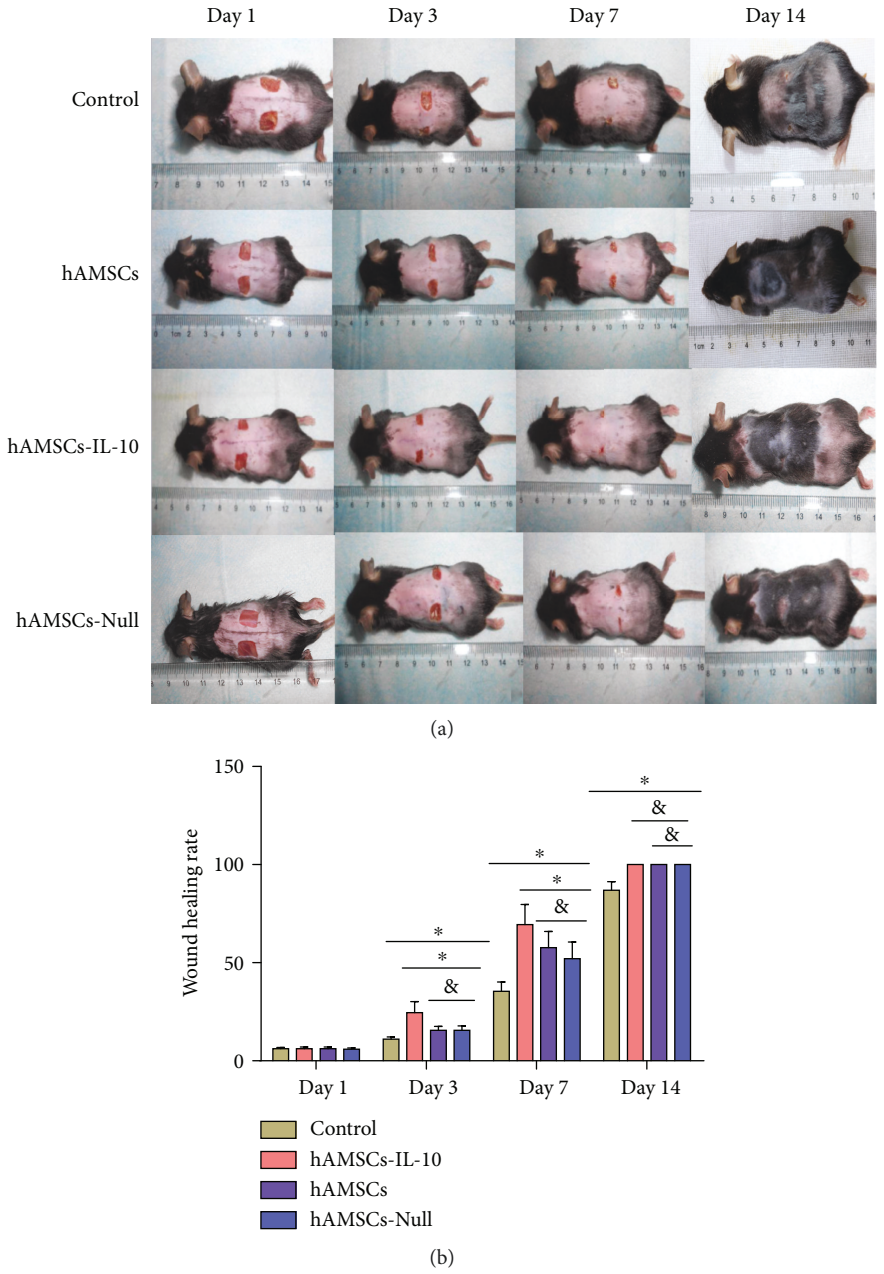

FIGURE 3: Injection of hAMSCs-IL-10 into subdermal periwound edges promoted wound healing. (a) Representative images of wound healing at indicated time points after cell transplantation. (b) Histogram of the healing rate of wounds at indicated time points with statistical analysis. Wound healing in the hAMSCs-IL-10 group was more rapid than that in the other three groups. $^{*}P < 0.05$ and $^{\&}P > 0.05$.

wound healing, whereas the control group still had unhealed wounds (Figures 3(a) and 3(b)).

3.4. IL-10-hAMSCs Had Anti-inflammatory Effects during the Healing Process. H&E staining of periwound skin sections revealed a large number of inflammatory cells infiltrating in all groups on day 3. The total number of inflammatory cells in the hAMSCs-IL-10 group was significantly lower than in the other three groups. On day 7, the number of inflammatory cells in the hAMSCs-IL-10, hAMSCs-Null, and hAMSC groups was reduced; the hAMSCs-IL-10 group showed the fewest inflammatory cells infiltrating, and many inflammatory cell infiltrations were seen in the control group. On day 14, the control group had little inflammatory cell infiltration, and almost no inflammatory cells were observed in the hAMSCs-IL-10, hAMSCs-Null, and hAMSC groups (Figures 4(a) and 4(b)).

We detected levels of the main anti-inflammatory cytokine IL-10 and proinflammatory factors IL-6 and TNF-α by ELISA and qPCR. The anti-inflammatory cytokine and proinflammatory factors peaked on day 3 and gradually decreased thereafter (Figure 5(a)). The level of IL-10 in the hAMSCs-IL-10 group was significantly higher than in the other three groups. The levels of IL-6 and TNF-α in the

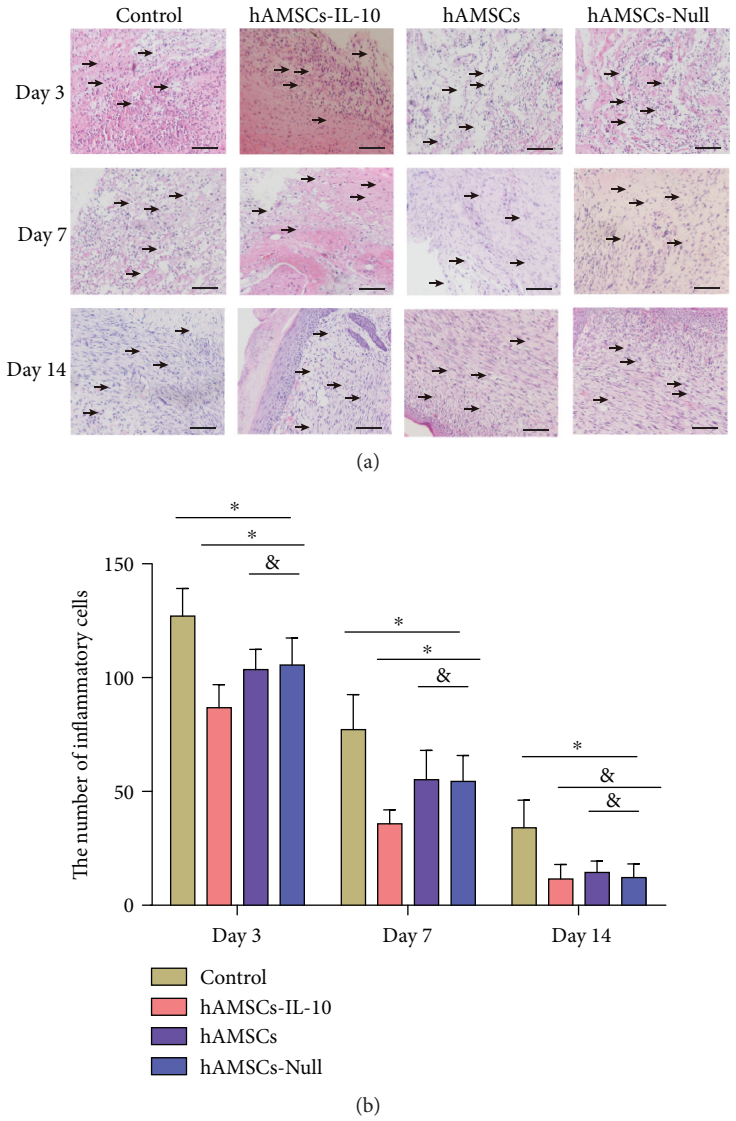

Figure 4: Inflammatory cell infiltration of periwounds. (a) Histology of inflammatory cell infiltration in the dermis, showing decrease in hAMSC-treated groups on days 3, 7, and 14 compared with controls. The hAMSCs-IL-10 group had the lowest infiltration of inflammatory cells. No significant difference was seen between the hAMSCs-Null and hAMSC groups. Arrows indicate inflammatory cells. (b) Quantification of inflammatory cells in the dermis by groups at indicated time points. Infiltration of inflammatory cells into the dermis decreased after treatment with hAMSCs-IL-10 compared with hAMSCs, hAMSCs-Null, or control on days 3 and 7 and decreased with hAMSCs and hAMSCs-Null compared with the control. No significant differences among the cell groups were seen in inflammatory cell infiltration on day 14. $^{*}P < 0.05$ and $^{\&}P > 0.05$. Scale bar = 500 μm.

hAMSCs-IL-10 group were significantly lower than in the other three groups (Figure 5(a)). The levels of IL-6 and TNF-α were lower, and that of IL-10 was higher in the hAMSCs and hAMSCs-Null groups compared with controls on days 3, 7, and 14. No significant differences between the hAMSCs and hAMSCs-Null groups were seen in expression of inflammatory factors. The expression of IL-10, IL-6, and TNF-α was confirmed by qPCR (Figure 5(b)). The expression of the relative mRNA levels of IL-10, IL-6, and TNF-α was consistent with the ELISA results.

3.5. IL-10-hAMSCs Upregulated Expression of Angiogenic Factors and Promoted Angiogenesis. At day 7 after cell transplantation, wound neovascularization occurred in all four groups. Neovascularization was quantified by counting microvessels. Compared to the control group, the hAMSCs-IL-10, hAMSCs-Null, and hAMSC groups had more microvessels. Also, the number of microvessels in the hAMSCs-IL-10 group was significantly higher than that in the hAMSCs-Null and hAMSC groups (Figures 6(a) and 6(b)).

To determine the potential effect of hAMSCs-IL-10 on wound angiogenesis, the levels of angiogenic factors VEGF and bFGF in cutaneous wounds were examined by ELISA. The expression levels of VEGF and bFGF in hAMSCs-IL-10, hAMSCs, and hAMSCs-Null groups were significantly higher than that in the control group, and hAMSCs-IL-10 had the highest expression of VEGF and bFGF. The hAMSCs-Null and hAMSC groups showed no significant

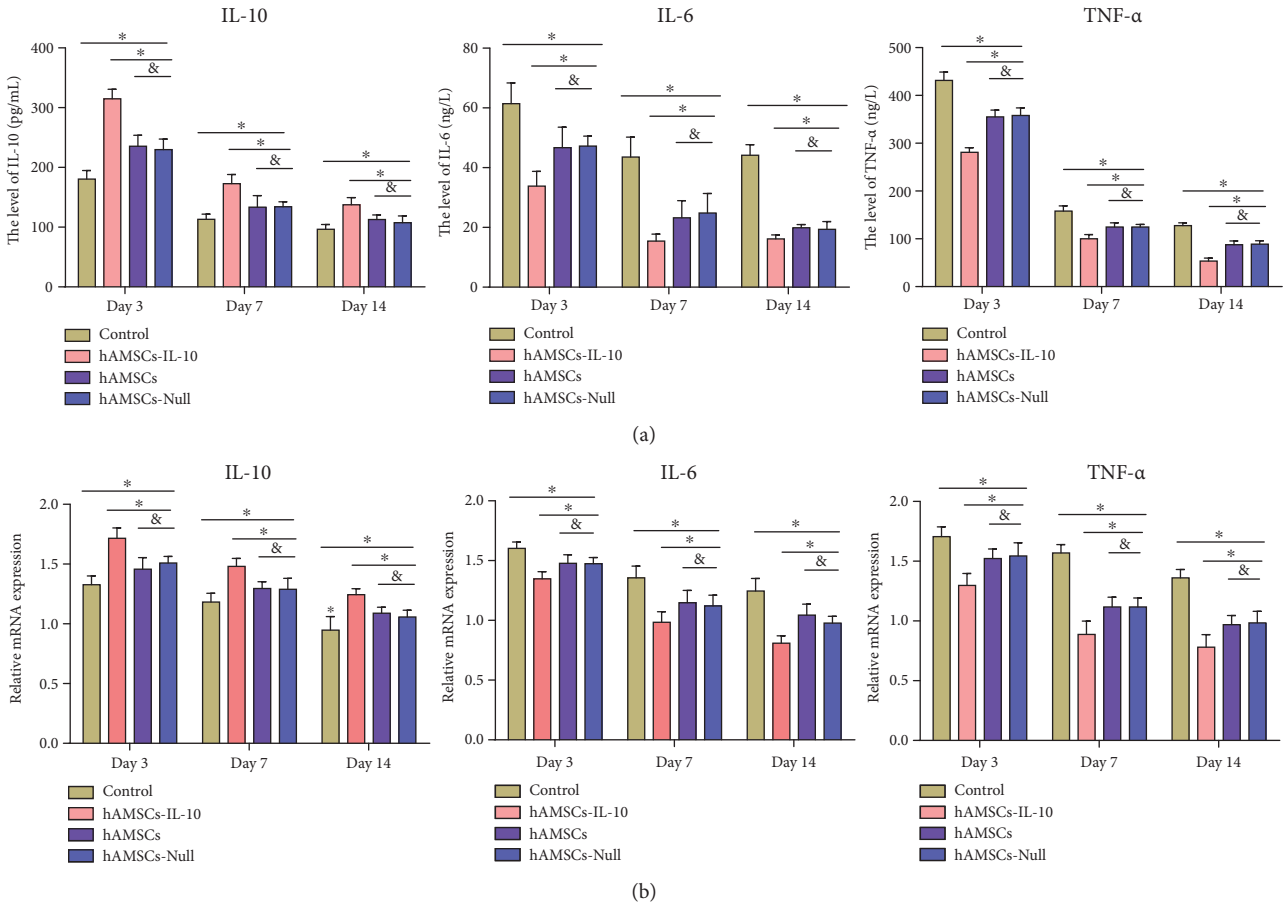

Figure 5: Expression of inflammatory factors. (a) ELISA of inflammatory factor expression by groups at indicated time points. The hAMSCs-IL-10 group showed the lowest expression of proinflammatory factors IL-6 and TNF-α and the highest expression of anti-inflammatory cytokine IL-10 on days 3, 7, and 14 compared with the hAMSCs, hAMSCs-Null, and control groups. Expression of proinflammatory factors IL-6 and TNF-α decreased after treatment with hAMSCs or hAMSCs-Null compared with the control on days 3, 7, and 14. Expression of anti-inflammatory cytokine IL-10 increased after treatment with hAMSCs or hAMSCs-Null compared with the control on days 3, 7, and 14. No significant differences among the hAMSCs and hAMSCs-Null groups were seen in expression of inflammatory factors ($n = 3$). (b) qPCR of inflammatory factor expression, in accord with ELISA results ($n = 3$). $^{*}P < 0.05$ and $^{\&}P > 0.05$.

difference (Figure 7). These results indicated that hAMSCs-IL-10 increased wound angiogenesis.

3.6. IL-10-hAMSCs Enhanced Proper ECM Events during Healing. ECM synthesis is an essential process of wound healing, and collagen is the main component of the ECM. To investigate the possible effect of hAMSCs-IL-10 on ECM production and remodeling during the healing process, accumulation of collagen was analyzed by Masson trichrome staining. All three hAMSC treatment groups had significant upregulation of collagen accumulation in skin compared with the control group on day 7 (Figures 8(a) and 8(b)). The IL-10-hAMSC group also showed higher collagen accumulation than the other groups, and collagen was arranged regularly. On day 14, collagen accumulation in the control group was significantly higher than in the other three groups, and collagen was arranged irregularly. The IL-10-hAMSC group showed a significantly lower collagen accumulation than the hAMSCs-Null and hAMSC groups.

MMP and TIMPs are important in ECM remodeling. TGF-β1 regulates the expression of MMP-1 and TIMP-1. We used ELISA to analyze the expression of TGF-β1, MMP, and TIMPs at different time points after cell transplantation. Compared to the control group, the hAMSCs-IL-10, hAMSCs, and hAMSCs-Null groups showed significant upregulation of TGF-β1, and the hAMSCs-IL-10 group showed the highest expression on day 7 (Figure 9(a)). The results were reversed on day 14, and expression of TGF-β1 in the control group was significantly higher than that in the other three groups with the hAMSCs-IL-10 group showing the lowest expression (Figure 9(a)). On day 7, expression of MMP-1 in the hAMSCs-IL-10, hAMSCs, and hAMSCs-Null groups showed significant downregulation compared with that in the control group, and the hAMSCs-IL-10 group had the lowest level. This result was reversed on day 14, with the expression of MMP-1 in the hAMSCs-IL-10 group significantly higher that than in other groups (Figure 9(b)). The expression of TIMP-1 was significantly increased at days 7 and 14 in wounds treated with hAMSCs-IL-10, hAMSCs, or hAMSCs-Null compared with the control group, and the hAMSCs-IL-10 group showed the highest level (Figure 9(c)). The ratio of

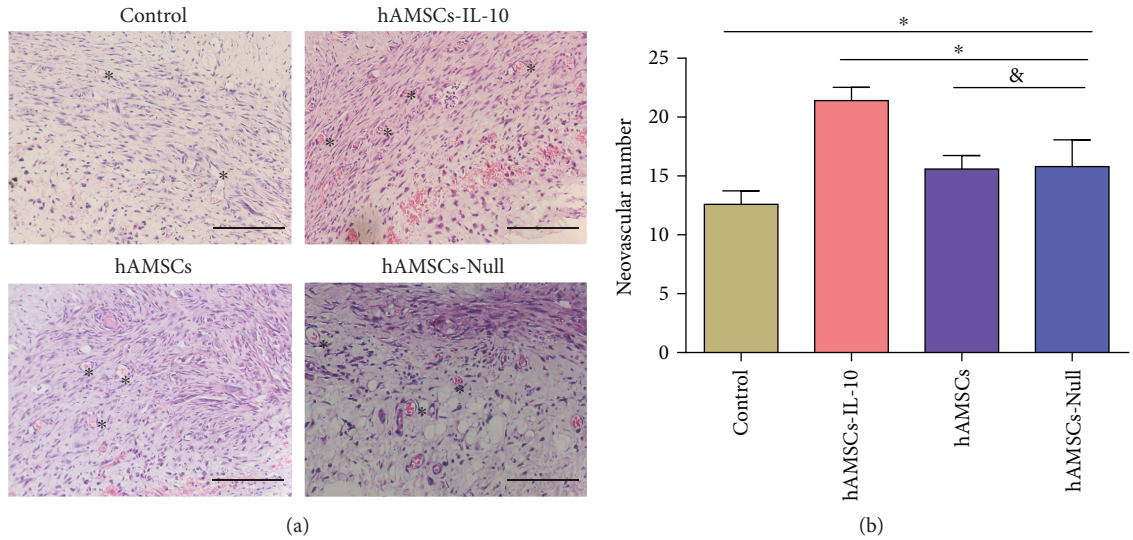

Figure 6: Effects of hAMSCs on wound neovascularization on day 7 after cell transplantation. (a) H&E staining showed more new blood vessels in the hAMSCs-IL-10, hAMSCs, and hAMSCs-Null groups than in the control group. The hAMSCs-IL-10 group had the most new blood vessels, and the hAMSCs-Null and hAMSC groups were not significantly different. *New blood vessel. Scale bar = 500 μm. (b) Quantification of new blood vessels in the dermis by groups on day 7. The number of new blood vessels in the dermis increased after treatment with hAMSCs-IL-10 compared with hAMSCs, hAMSCs-Null, or control on day 7 and increased with hAMSCs and hAMSCs-Null compared with the control. No significant differences between the hAMSCs and hAMSCs-Null groups were seen in vascularization on day 7. $^{*}P < 0.05$ and $^{\&}P > 0.05$.

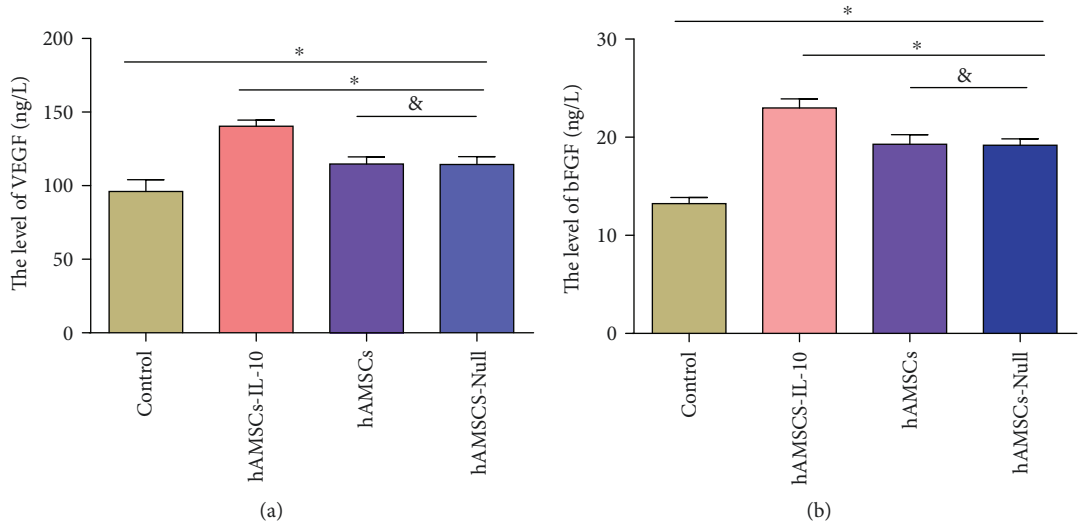

Figure 7: Expression of angiogenic factors on day 7 after cell transplantation by ELISA. ELISA showing comparison with controls: the hAMSCs-IL-10, hAMSCs, and hAMSCs-Null groups had higher expression, and hAMSCs-IL-10 had the highest expression of VEGF and bFGF. The hAMSCs-Null and hAMSC groups showed no significant differences ($n = 3$). $^{*}P < 0.05$ and $^{\&}P > 0.05$.

TIMP-1/MMP-1 was highest in the hAMSCs-IL-10 group on day 7 and lowest on day 14 (Figure 9(d)).

3.7. Colonization and Survival of hAMSCs In Vivo. To observe the colonization and survival of hAMSCs *in vivo*, hAMSCs were stained with CM-DiI, and hAMSCs-IL-10 and hAMSCs-Null expressed green fluorescence protein, 14 days after cell transplantation. Wound skin was fixed, and frozen sections were prepared and examined under fluorescence microscopy. Group fluorescence distributions indicated that hAMSCs, hAMSCs-IL-10, and hAMSCs-Null were colonized and survived in tissues (Figure 10).

4. Discussion

Although bone marrow stem cells (BMSCs) have been extensively studied for wound healing, their collection is associated with invasive procedures and amounts that are relatively low. The number of BMSCs falls as donor age increases [12]. The search for easily accessible and noninvasive procedures to

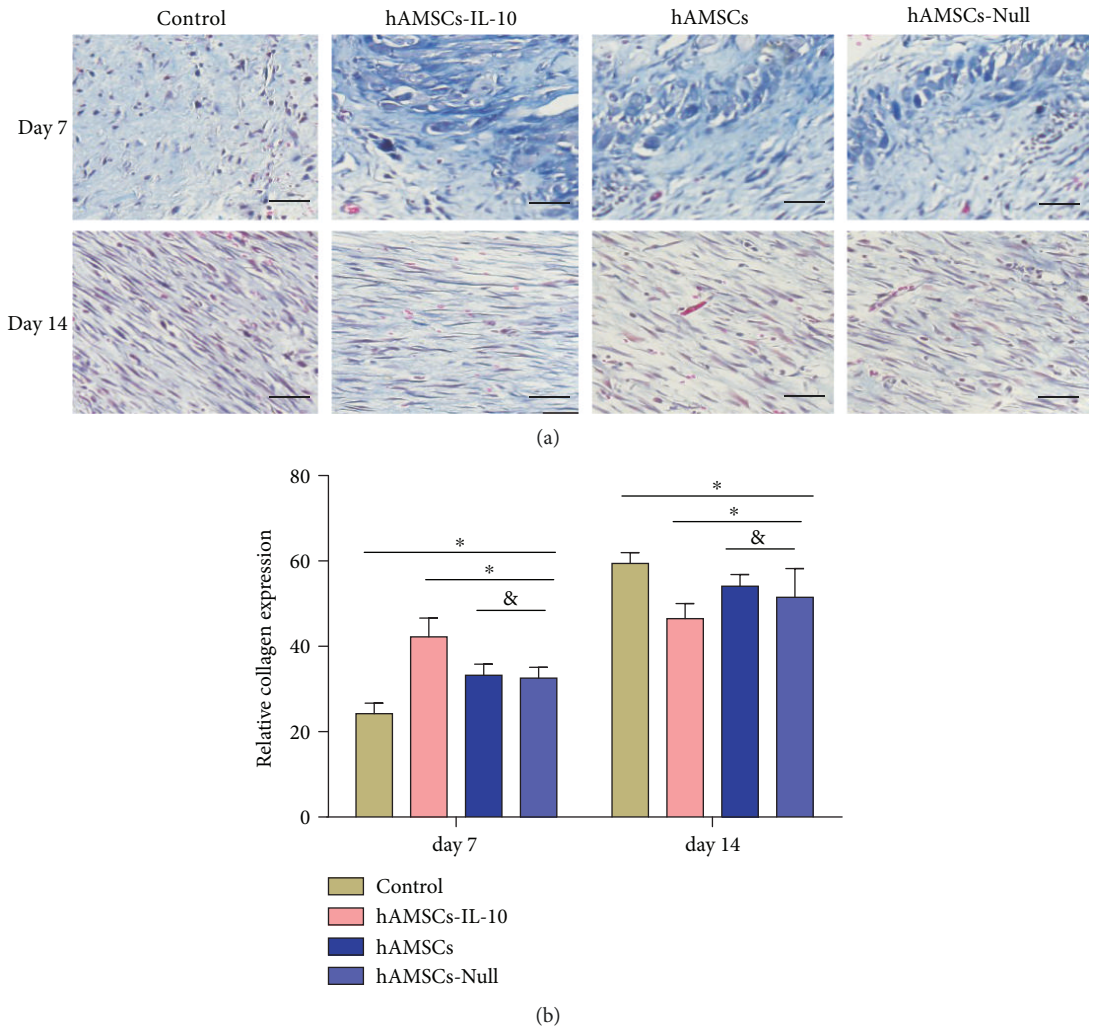

Figure 8: Collagen accumulation in wounds on days 7 and 14. (a) Masson staining of collagen deposition in the dermis showed increases in the hAMSCs-IL-10, hAMSCs, and hAMSCs-Null groups on day 7 compared with the control group; the hAMSCs-IL-10 group had the most collagen, and collagen was arranged regularly; the hAMSCs-Null and hAMSC groups were not significantly different. On day 14, the control group had the highest collagen deposition, and collagen was arranged irregularly. The hAMSCs-IL-10 group had the lowest collagen deposition. Scale bar = 50 μm. (b) Quantification of collagen synthesis in wound skin by groups at indicated time points. $^{*}P < 0.05$ and $^{\&}P > 0.05$.

obtain MSCs has focused on other human tissues, such as the placenta. hAMSCs are isolated from the amniotic membrane of human medical waste material with minimal ethical problems. Compared with MSCs from other sources, hAMSCs are isolated by simple enzymatic digestion procedures from a single amnion for more than 10^7 cells with high proliferative capacity [13]. As hAMSCs express low levels of classical MHC-I and do not express MHC-II, they survive in immunocompatibility mismatched allogeneic transplant recipients. They are promising for applications in the field of regenerative medicine [14].

IL-10 has received attention because of its potent multiple biological effects. It is a pivotal factor in wound healing [8–10]. IL-10 delivery by scaffold materials such as collagen-silica [15], polycaprolactone (PCL) [16], transgene adjuvant D-mannose [17], and viruses [18] has been investigated. However, IL-10 has a short half-life *in vivo* [19]. Spatiotemporal control over bioactive molecule release can achieve optimal efficacy [19]. MSCs can regulate paracrine signals by sensing wound microenvironment, and IL-10 is a key factor in immunomodulatory properties of MSCs [20, 21]. In addition to having biological effects that promote wound regeneration healing, MSCs are an attractive vehicle for gene delivery for regeneration medicine [4, 6, 7]. Therefore, an excellent strategy would be MSCs as a vehicle for IL-10 delivery for wound regeneration healing. However, the biological effects of IL-10 delivery by MSCs in wound healing remain unclear.

In this study, we used the IL-10 gene to modify hAMSCs to exert synergistic effects such as modulating inflammation, enhancing angiogenesis, and regulating ECM remodeling, to promote regenerative wound healing. We found that hAMSCs-IL-10 secreted IL-10 at higher levels than unmodified hAMSCs. Wounds treated with hAMSCs-IL-10 showed more rapid wound closure and better wound-healing qualities.

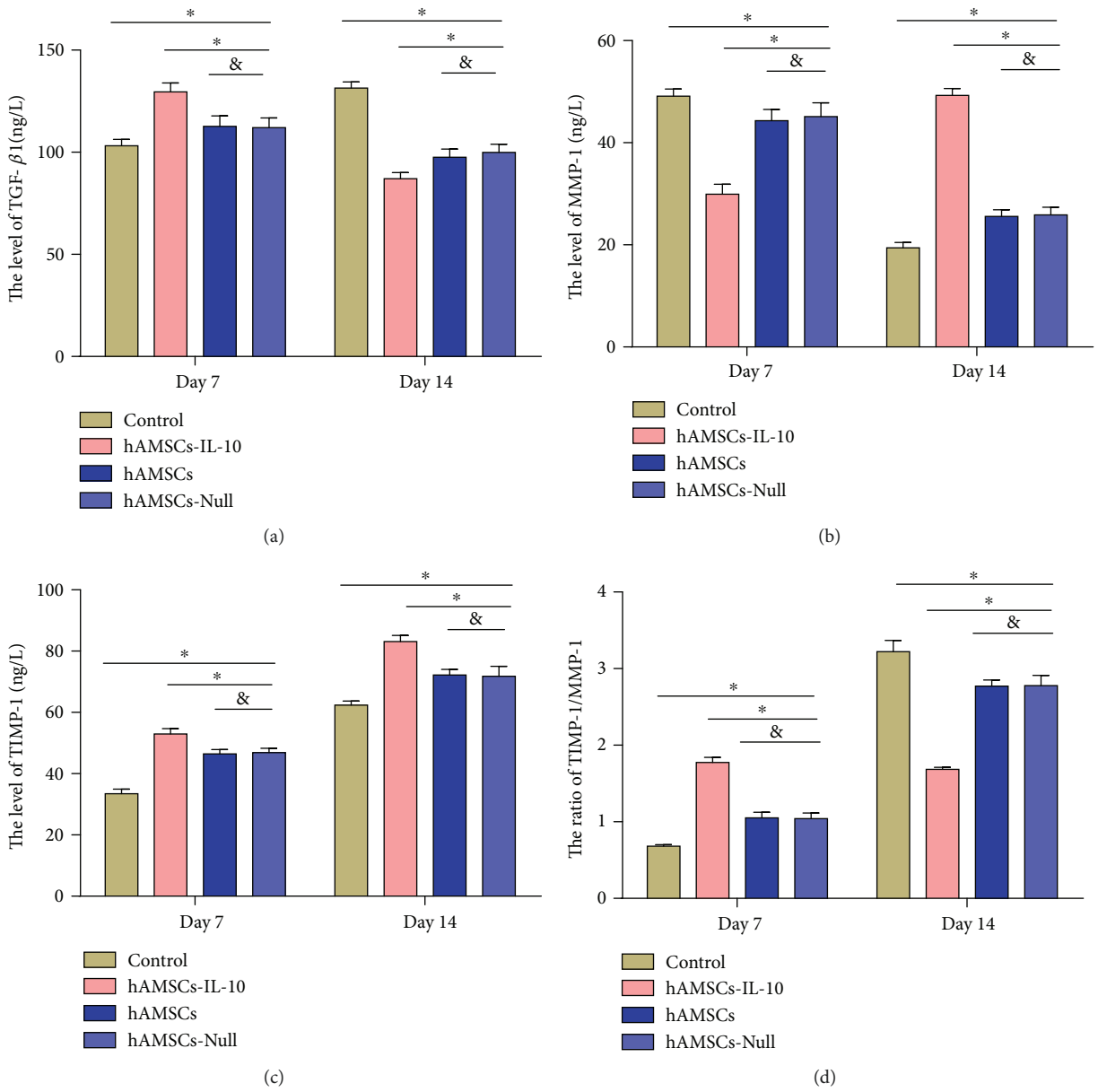

Figure 9: Effects of hAMSCs on wound ECM remodeling on days 7 and 14 after cell transplantation. (a) TGF-β1 expression by groups at indicated time points. The hAMSCs-IL-10 group showed the highest expression of TGF-β1 on day 7 compared with the hAMSCs, hAMSCs-Null, and control groups. On day 14, expression of TGF-β1 in the control group was significantly higher than that in the other groups with the hAMSCs-IL-10 group showing the lowest expression. (b) MMP-1 expression by groups at indicated time points. The hAMSCs-IL-10 group showed the lowest expression of MMP-1 on day 7 compared with the hAMSCs, hAMSCs-Null, and control groups. On day 14, expression of MMP-1 in the hAMSCs-IL-10 group was significantly higher than that in other groups. (c) TIMP-1 expression by groups at indicated time points. Expression of TIMP-1 was significantly increased at days 7 and 14 in wounds treated with hAMSCs-IL-10, hAMSCs, or hAMSCs-Null compared with the control group; the hAMSCs-IL-10 group showed the highest level. (d) TIMP-1/MMP-1 ratio was highest in the hAMSCs-IL-10 group on day 7 and lowest on day 14. $^{*}P < 0.05$ and $^{\&}P > 0.05$.

Inflammation is an essential, nonspecific, innate immune response involving the breakdown of tissue and cleanup of cellular, extracellular, and pathogenic debris. However, in the presence of an external noxious stimulus that causes tissue damage, inflammation can become prolonged and heightened. IL-6 is a proinflammatory cytokine that exerts multiple effects in direct opposition to the anti-inflammatory cytokine IL-10, such as increasing inflammatory cell infiltration [22]. Evidence is provided by the formation of scars with addition of IL-6 in the fetus at a gestational age that should heal without scarring [23]. With IL-10 functions in fetal regenerative wound healing, these findings have led to the "cytokine hypothesis" that proposes that fetal tissue is permissive of regenerative healing due to relatively elevated levels of anti-inflammatory cytokine expression compared with proinflammatory cytokines, leading to an anti-inflammatory wound milieu [1, 2]. TNF-α, also an important proinflammatory cytokine, is one of the implicated molecules and has a key function in

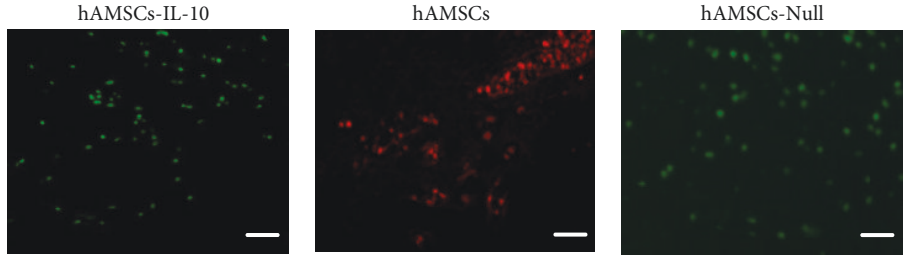

Figure 10: Fluorescent image of frozen sections of wound skin. When exposed to green light, transplanted hAMSCs showed red fluorescence. When exposed to blue light, hAMSCs-IL-10 and hAMSCs-Null expressed green fluorescence. Labeled cells concentrated in subcutaneous tissues on day 14 after cell transplantation, indicating that hAMSCs-IL-10, hAMSCs, and hAMSCs-Null survived after transplantation. Scale bar = 500 μm.

inflammation and subsequent wound healing [24]. Our results showed that hAMSCs-IL-10 attenuated the inflammatory response of local wounds by decreasing inflammatory cell infiltration as well as by production of proinflammatory cytokines IL-6 and TNF-α. hAMSCs-IL-10 administration clearly showed that anti-inflammatory effects were enhanced by synergistic effects of hAMSCs and IL-10.

VEGF is one of the most potent proangiogenic factors. It is secreted by keratinocytes and macrophages and acts to promote the proliferation of vascular endothelial cells [25]. bFGF is a strong angiogenic factor that stimulates the migration and proliferation of vascular endothelial cells and facilitates capillary formation. During wound healing, VEGF and bFGF synergistically stimulate endothelial cell proliferation, promote vascularization, and accelerate the process of wound healing [26]. Several studies demonstrated that MSCs in wound areas can secrete cytokines such as VEGF, bFGF, and PDGF, resulting in enhanced angiogenesis and wound healing. Some soluble factors secreted from MSCs induce endothelial cell survival, vascular branching, and pericyte recruitment [27]. Our study found that hAMSCs-IL-10 upregulated VEGF and bFGF and increased the density of microvessels in local wounds. We hypothesized that hAMSCs-IL-10 accelerated wound healing by paracrine VEGF and bFGF to increase wound angiogenesis.

TGF-β1 has multiple functions in the process of wound healing. First, TGF-β1 is involved in the regulation of the inflammation response. In the early stage of wound healing, the level of TGF-β1 is low, promoting the migration of neutrophils and macrophages. In the middle stage of wound healing, the level of TGF-β1 increases rapidly, which can suppress the migration and activation of lymphocytes and macrophages, and result in decreased inflammation [28]. Second, TGF-β1 is involved in the regulation of proliferation and differentiation of fibroblasts through autocrine and paracrine effects. Low concentrations of TGF-β1 promote proliferation and differentiation of fibroblasts and result in increased collagen deposition. High concentrations of TGF-β1 inhibit proliferation and differentiation of fibroblasts and decreased collagen deposition [28]. Third, TGF-β1 is involved in the regulation of MMP-1 and TIMP-1 in the ECM. MMP-1 is a major factor in the degradation of collagen in ECM. TIMP-1 is a special inhibitor of MMP-1. MMP-1 and TIMP-1 and constitutes a compact complex with proportion in dynamic equilibrium to regulate the degradation and deposition of ECM [29, 30]. High levels of TGF-β1 downregulate expression of MMP-1, resulting in an increase in TIMP-1 response. Eventually, the deposition of collagen increases and degradation of ECM reduces, resulting in scar hyperplasia [31]. Our results showed that hAMSCs upregulated the expression of TGF-β1 in the early and middle stages of wound healing and downregulated the expression of TGF-β1 in the middle and late stages of wound healing. In the middle and late stages of wound healing, hAMSCs increased the expression of MMP-1 and TIMP-1 and reduced the ratio of TIMP-1/MMP-1. By reducing inflammation and fibrosis, hAMSC administration accelerated wound healing and alleviated scar formation. IL-10 enhanced these effects of hAMSCs, and hAMSCs-IL-10 had stronger biological effects than hAMSCs.

hAMSCs-IL-10 transplantation promoted wound healing and improved healing qualities more effectively by upregulating expression of IL-10, modulating inflammation, enhancing angiogenesis, promoting granulation tissue formation, and regulating ECM remodeling. These data may thus provide a theoretical foundation for clinical administration of hAMSCs-IL-10 in cutaneous wound healing and skin regeneration. In this study, at 14 days after cell transplantation, we observed that cells were colonized and survived in tissues. However, the fate of hAMSCs after a long time is worth further investigation. Tracking and verification are complex and with remaining questions to be answered, such as cell fusion before the final destination becomes clear.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

All authors agree with the manuscript content. Dali Wang and Shune Xiao designed the experiments; Guangtao Huang

performed the cellular biology experiments and statistical analysis. Zhiyuan Liu and Kaiyu Nie conducted the animal assay. Chengliang Deng provided many suggestions during manuscript preparation, and Shune Xiao wrote the manuscript. Shune Xiao and Guangtao Huang contributed equally and are co-first authors.

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