Stem Cells International

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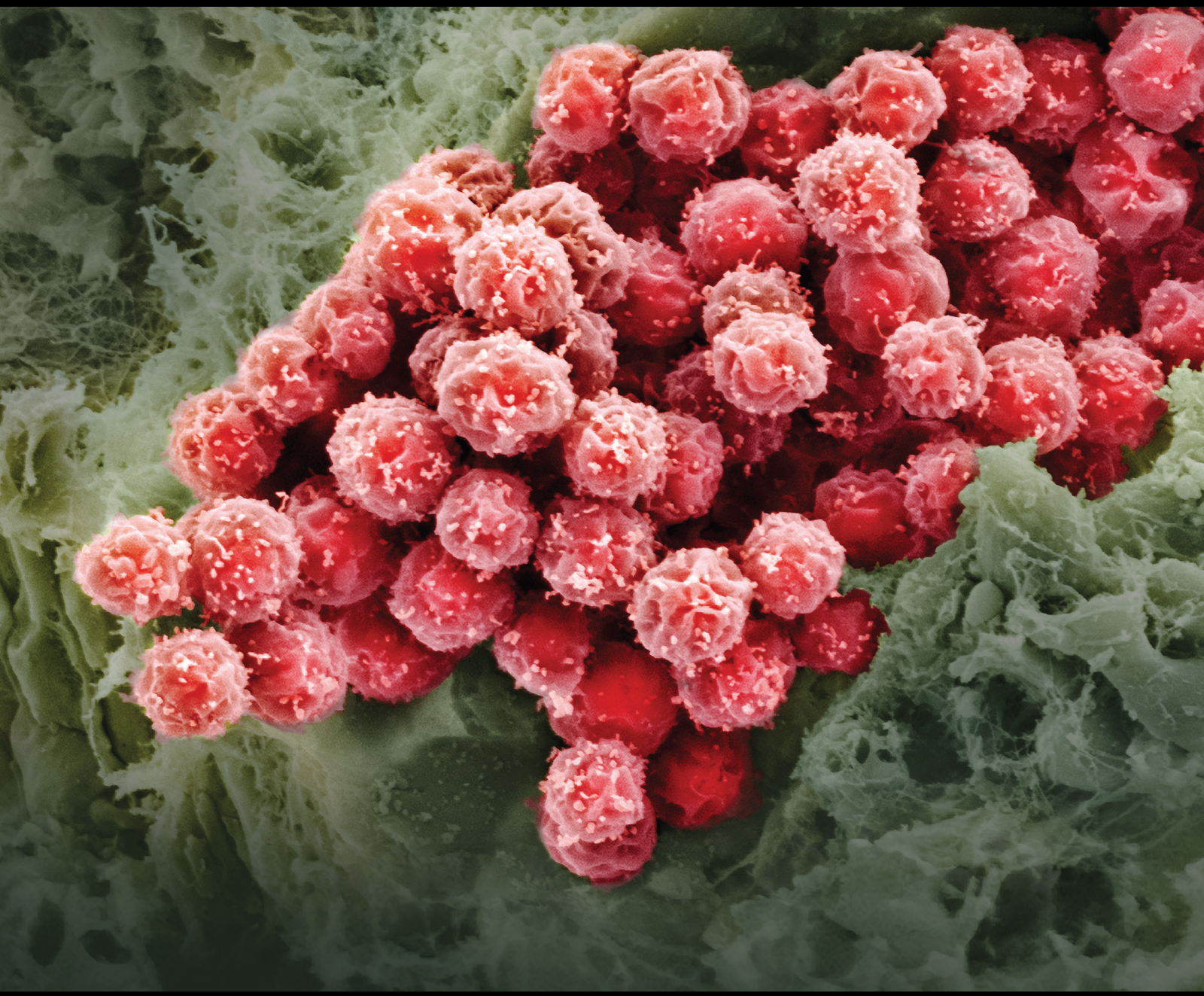

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Review Article

Metabolic Glycoengineering: A Promising Strategy to Remodel Microenvironments for Regenerative Therapy

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Cell-based regenerative therapy utilizes the differentiation potential of stem cells to rejuvenate tissues. But the dynamic fate of stem cells is calling for precise control to optimize their therapeutic efficiency. Stem cell fate is regulated by specific conditions called "microenvironments." Among the various factors in the microenvironment, the cell-surface glycan acts as a mediator of cell-matrix and cell-cell interactions and manipulates the behavior of cells. Herein, metabolic glycoengineering (MGE) is an easy but powerful technology for remodeling the structure of glycan. By presenting unnatural glycans on the surface, MGE provides us an opportunity to reshape the microenvironment and evoke desired cellular responses. In this review, we firstly focused on the determining role of glycans on cellular activity; then, we introduced how MGE influences glycosylation and subsequently affects cell fate; at last, we outlined the application of MGE in regenerative therapy, especially in the musculoskeletal system, and the future direction of MGE is discussed.

1. Introduction

The need to regenerate or replace impaired tissues is rising nowadays, owing to the extended lifespan and the attendant degenerative diseases, as well as trauma and tumors [1]. Exogenous stem cells provide convenience to obtain as well as broad differentiation attributes. They not only can propagate under a static state but also can be induced to differentiate towards specialized cells or tissues with proper stimuli, aiming to rejuvenate the degenerated tissues back to their normal functional state [2, 3].

But the stem cells also bring us a question: how to maximize their regenerative efficiency after transplantation? The fate of stem cells can be more dynamic than we expected including adhesion [4], migration [5], proliferation, and apoptosis [6]. Besides, the survival rate of stem cells after transplantation also remained unsatisfactory.

The cellular microenvironment can be considered as a fundamental entry point for these obstacles above, because the decision of cell fate is highly regulated by matrix mechanical cues [7], biochemical factors [8], and other manners like intercellular crosstalk [9]. Researchers have endeavored to grasp the characteristics of the cellular microenvironment, in which cell-surface modification might be an ideal approach to regulate it.

In general, the cell membrane provides a platform carrying proteins or molecules that assist cells to conduct essential signals from external stimuli. Modification of cell membrane can display heterologous proteins on the surface, thereby inducing cellular responses and regulating biological behaviors [10].

The major technologies of cell-surface modification include hydrophobic membrane insertion [11], chemical conjugation [12], liposome fusion [13], metabolic pathways [14], enzymatic modification [15], and genetic engineering [16]. Among them, genetic engineering is the most widely used approach which incorporates materials into the genome of cells and then encodes target receptors onto the surface, such as cargocytes [17] and chimeric antigen receptor (CAR) T cells [18, 19].

Although genetic engineering could be a robust strategy to modify membranes, it is also associated with some drawbacks: (1) the process is time-consuming; (2) genetic transfection using viral vectors may cause unpredictable risks; (3) the irreversible modification may raise safety concerns for clinical applications; (4) moreover, not all cell types can adapt to genetic alteration without side effects, particularly in stem cells [20, 21].

In contrast, metabolic glycoengineering (MGE) is a safe and reversible strategy for membrane modification using nongenetic methods. Unlike nucleic acid or proteins, the cell-surface glycans are not directly encoded by genes. Biosynthesis of glycans (glycosylation) is determined by intra- or extracellular factors such as substrate transferases, signal transduction, and metabolic pathways [22, 23]. MGE technique is aimed at manipulating glycosylation and cellular metabolism to increase the expression levels of natural glycans and, more importantly, to install nonnatural monosaccharides into cell-surface glycoconjugates, such as ketone-, azide-, thiol-, or alkyne-modified glycans [24, 25].

Since MGE exploits the inherent natural metabolic pathway of cells, the process of modification barely interferes with other cellular functions—sort of like "silent" labeling [26]. Meanwhile, the MGE strategy also has other advantages as (1) easy but efficient process by simply coculturing cells with metabolic precursors; (2) exhibits noncytotoxicity even under high concentration of treatment; (3) applicable to almost all cell types; (4) nonpermanent modification that allows controlled reversal; and (5) the diversity of sugar analogs and bioorthogonal click chemistry endows MGE with numerous choices for membrane modification.

Therefore, by modifying surface glycans' structure or expression flux to decorate the cell membrane, MGE can circumvent the limitations associated with other strategies like genetic engineering.

In this review, (1) we firstly illustrated the significant role of cell-surface glycans in cell microenvironments; (2) then, we described how metabolic glycoengineering (MGE) influences cellular behaviors, and we exemplified MGE's effect on cell fate control; (3) finally, we outlined the application of MGE in regenerative medicine with a focus on the musculoskeletal system, and the future direction is also discussed.

2. Cellular Microenvironment, Extracellular Matrix, and Cell-Surface Glycans

2.1. Microenvironment and Extracellular Matrix: Shelters for Cells. Cell microenvironments are small zones around cells that can be defined as the intercellular substance containing dynamic body fluid components [27]. Particularly, stem cells are residing in specialized microenvironments that donated as "stem cell niches," which provide stem cells with static status and low-energy-consuming conditions to maintain their balance between self-renewal and differentiation [28].

The cell microenvironments consist of a set of elements that influence cellular activities (Figure 1), which can be mainly classified into the following types: extracellular matrix (ECM), adjacent cells (both homotypic or heterotypic), mechanical forces, proteolytic enzyme factors, and inflammatory cytokines [29]. And ECM represents the major component of the stem cell niche.

On the one hand, ECM provides cells with physical support, cytoskeletal structure, and transduction of physiological signals. The ECM components such as glycosaminoglycans (GAG) and adhesive molecules are maintaining the stability of cells, and the proper adhesion to ECM is essential for the survival of adjacent cells [30]. Partial geometric control of cell growth by spreading is also a basic mechanism for the developmental modulation of ECM [31].

On the other hand, microenvironments are rather dynamic than stuck in a rut. Cells remodel their microenvironments by altering the secretion of ECM components. Each kind of tissue creates a unique ECM composition, which is responsible for tissue-specific behaviors. The high affinity between stem cells and ECM can selectively influence the cell differentiation towards specific orientations, according to respective ECM components [32, 33] (e.g., fibroblasts to fibronectin, chondrocytes to type II collagen, or endothelial cells to laminin).

Conversely, without effective adhesion to ECM, cells tend to start the procedure of cell death such as apoptosis and necroptosis [34]. This phenomenon was initially reported in epithelial cells that the disrupted cell-matrix interaction will result in cell anoikis (a special type of apoptosis) [35]. However, the loss of function of the anoikis signals will also lead to another polarization, cancerous differentiation and metastasis [36], which reminds us about the injection of embryonic stem cells (ESCs) that generally results in teratoma formation [37].

Henceforth, considering the pluripotent of stem cells, it is critical to ensure desired interactions between cells and ECM. There could be many variations that we can precisely control for altering the specific characteristics of the microenvironment. And cell-surface glycan could be a major participant since sugars are ubiquitously present in all classes of cells, where they function as sources of energy, regulators of signaling, and participants of metabolic activity.

2.2. Cell-Surface Glycans: The Active Determinants of Microenvironment and Stem Cell Fate. Cellular glycans can be found in proteoglycans, glycoproteins, and glycolipids. Among all these glycoconjugates, cell-surface glycans (also called glycocalyx) are polysaccharides wrapping around the surfaces of all mammalian cells and participating in cell-cell [38] and cell-matrix [39] interactions (Figure 1). Like correspondences, these interactions mark the function of a cell, specify how it communicates with its surroundings, and also influence immune response [40].

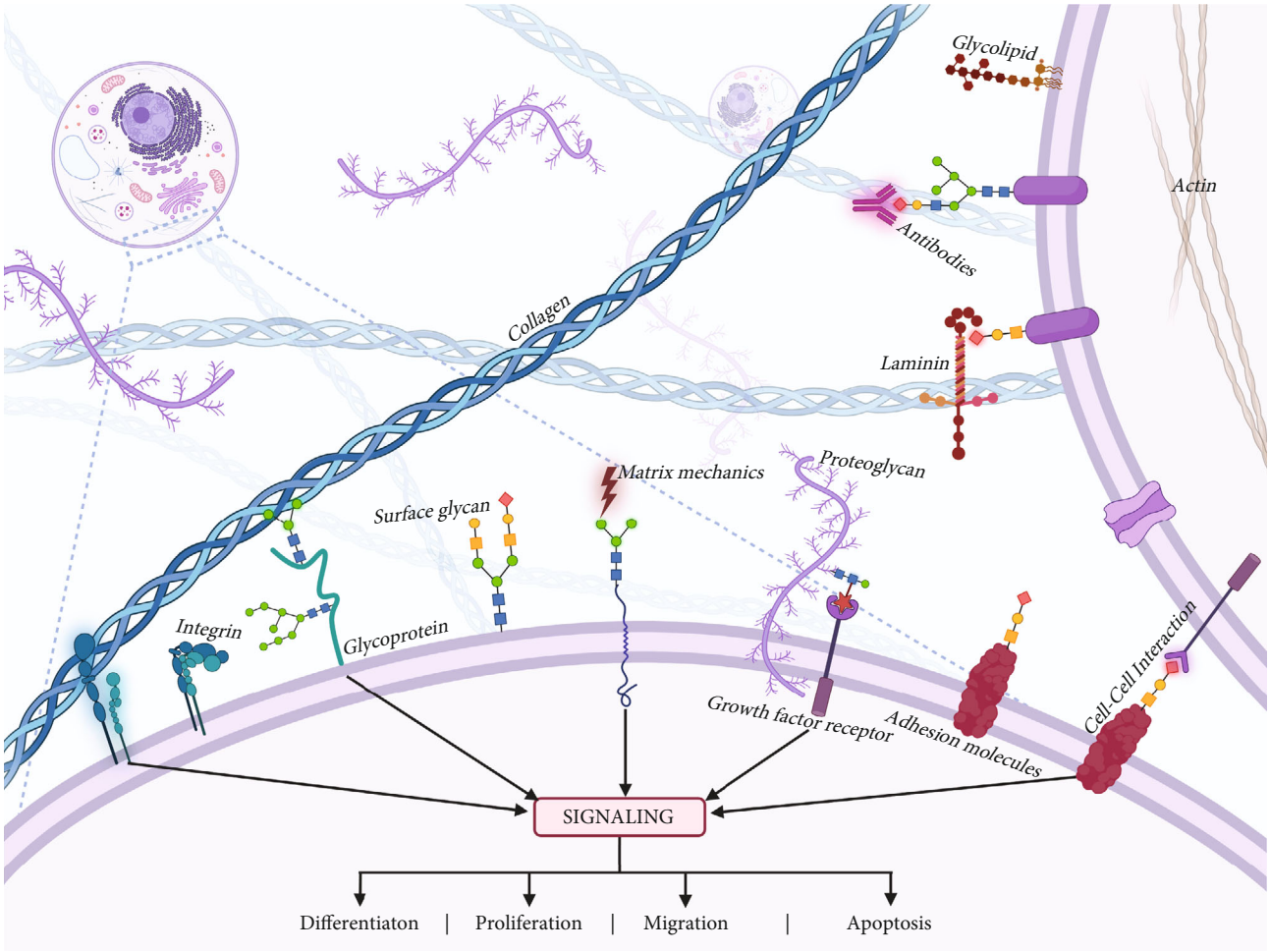

Figure 1: Interactions in the microenvironment. Cells reside in a dynamic environment consisting of ECM components, adjacent cells, mechanical forces, and various biological cues. The cell membranes offer platforms for cellular activities within their microenvironment, including molecule recognition, cell-cell interaction, and cell-matrix combination, which will, respectively, lead to different cell fates. Cell-surface glycans wrapped the membranes of cells and mediated those interactions in the microenvironment.

Glycosylation, the covalent attachment of glycans, is the most abundant posttranscriptional modification of proteins in nature [41]. O-Glycosylation and N-glycosylation are two major versions of protein glycosylation. O-Glycosylation involves the attachment of monosaccharides (N-acetylglucosamine) or polysaccharides (glycans) to threonine, serine, or tyrosine, while N-glycosylation added glycans to asparagine residues selectively [42]. Consequently, differences in structural composition conferred diversity of function to different glycosylated proteins.

For example, α-2-6-sialylated N-glycans, but not O-glycans, could be used as markers of the differential potential of mesenchymal stem cells [43]. Cell-surface N-glycans were also proved to influence the electrophysiological properties and differential fate of neural progenitor cells [44]. Moreover, the defect in glycosylation will lead to the disrupted adhesion of epithelial cells and then impair the cellular microenvironment [45]. The glycosylation can also influence cell fate directly. For example, both N- and O-glycosylation in cells functionally modulate the early steps of osteogenic differentiation of skeletal progenitor cells [46, 47].

Among the many glycosylation processes, sialylation is a significant one that happens on the terminal of mucin protein, mediated by sialyltransferases (ST) [48]. And cellular sialylation is essential in cell adhesion because the expression of integrin ligands is closely related to it [49, 50]. Integrin determines which ECM component cells would bind to during cell development and thus selectively affects the cell morphology [51]. Beyond integrins, the selectin family is another determinant cluster of adhesion that recognizes sialylation, while the sialylated Lewis X (sLeX) is the ligand for selectin [52, 53]. And some sialylated molecules also perform direct effects on the nervous system, with some gangliosides (GM3 and GD3) that control the early development by impacting cell growth and apoptosis [54]. Moreover, the precursors of sialyation were reported to act as signaling molecules that control the differentiation of neural cells [55]. In short, the function, stability, and metabolism of glycoproteins are dependent upon correct sialylation.

Cell-surface glycans are important factors that facilitate communication with the ECM and mediate signaling cascades and, consequently, make glycosylation or sialylation

an active determinant in the microenvironment, regulating cell fate in both direct and indirect manners.

3. Metabolic Glycoengineering: A "Silent" Method for Glycan Modification

The synthesis of cell-surface glycans is determined by substrate transferases and metabolic conditions. Herein, metabolic glycoengineering (MGE) is a nongenetic strategy for glycan modification based on metabolic precursors.

3.1. Overview of MGE. The main purpose of MGE is to increase the expression levels of natural glycans and install nonnatural monosaccharides into cell-surface glycoconjugates [24]. To put it in another way, MGE introduces various chemical groups into cellular glycan by artificially modified monosaccharides that bear unnatural functionalities (R-groups). While being incubated with mammalian cells, those monosaccharides can intercept the glycosylation pathways in cells, resulting in the submission of R-group-modified glycans on cell surfaces or secreted as glycoconjugate [56, 57].

Sialic acid, also known as neuraminic acid, is mainly located at the ends of the side chains of cell membrane glycoproteins, where it participates in numerous interactions between cell and microenvironment [25].

N-Acetylneuraminic acid (Neu5Ac) is the most common form of sialic acid in human cells while the N-acetyl-D-mannosamine (ManNAc) acts as the physiological precursor of all sialic acids. After ManNAc is absorbed into a cell as a precursor, it is converted to Neu5Ac with the help of specific sialyltransferases and will eventually be anchored to the residues of cell-surface sialic acid (Figure 2(a)).

The sialic acid pathway was the first glycosylation pathway to be utilized in MGE [58], and it is also the most commonly used pathway nowadays. The reason why the sialic acid pathway becomes a suitable choice for MGE is relevant to the remarkable substrate promiscuity of sialyltransferases [59, 60], which provides the possibility for the modified analogs to successfully intercept glycosylation pathways, resulting in the chemically modified sialic acid (Figure 2(a)). For example, N-propionyl-mannosamine (ManNProp) is an analog of ManNAc with a propionyl group on the N-acyl side chain (Figure 2(b)Ba), and the metabolism of ManNProp eventually submits N-propionyl-neuraminic acid (Neu5Prop) on the cell membrane surface [61].

3.2. Metabolic Precursors of Sialic Acid. Since Kayser et al. developed the 1st generation of ManNAc analogs (ManNProp, ManNBut, and ManNPent) in the 1990s [56], more than dozens of unnatural monosaccharides have been synthesized as appropriate precursors for MGE. Among all these ManNAc analogs, two major categories can be grouped: (i) aliphatic analogs and (ii) bioorthogonal analogs.

Aliphatic analogs are characterized by their N-acyl side chains which elongated with one or more methylene groups. Slight modifications of the sialic acid N-acyl side chain, such as the introduction of hydrophobic methylene, will cause significant impacts on specific cell-surface biological functionalities (Figure 2(b)Ba), including virus infection receptors [62], cell-surface differentiation markers [63], and cell proliferating regulation [64]. Thus, MGE based on aliphatic ManNAc analogs is aimed at bringing additional biological features to cells and then consequently influencing their behavior and fate.

Bioorthogonal analogs are synthesized with N-acyl side chains carrying reactive R-groups which are absent in biological systems but could be utilized for further chemical conjugations or reactions (Figure 2(b)Bb). Bioorthogonal chemistry enables the installation of artificial functionalities onto the cell surface, such as drugs [65], ligands [66], macromolecules [67], or fluorescent dyes [68]. For example, azide-functionalized N-acetylmannosamine (ManNAz) can produce azide groups on N-acyl side chains of sialic acid. Azide groups are absent from mammalian cells, but it holds bioorthogonal reactivity with most biofunctional groups. To be specific, based on the high efficiency of reactions between dibenzocyclooctyne (DBCO) and azide groups, certain DBCO-modified substances can be attached to these azide groups on cell surface, resulting in the combination that we expected. Theoretically, any functional substance of our interests can be installed onto sialic acid by using the two-step bioorthogonal reaction. This connecting process undergoes copper-free click chemistry, which is characterized by its linkage stability, biocompatibility, and noncytotoxicity.

Beyond sialic acid, other glycosylation pathways have also been exploited with the development of MGE, such as L-fucose [69], GlcNAc (N-acetylglucosamine) [70], and GalNAc (N-acetylgalactosamine) [71], which provide more opportunities for MGE's application in various fields. A comprehensive description of MGE can be seen in these review articles [26, 72, 73]. But the major effort in MGE continued to concentrate on the sialic acid pathway, due to its biological importance and the outstanding permissibility of sialyltransferases for nonnatural analogs.

In the next section, we will focus our sight on applications of MGE in inducing biological cellular response, particularly in cell fate control.

4. The Impact of MGE on Cell Fate

Considering the significance of glycocalyx in biological activities as well as the accessibility of sialic acid to be chemically modified, glycans can act as targets for controlling cell fate.

Evidence is adequate that sialic acid precursors can precisely influence cellular behaviors. For example, ManNBut could reversibly inhibit the expression of cell-surface polysialic acid (polySia) while ManNProp did not downregulate it, due to the shorter N-acyl side chain of ManNProp [74]. ManNProp and ManNBut only differ from 1 methylene unit (-CH3) in their terminal structure of the N-acyl side chain (Figure 2(b)Ba), but these two analogs tend to elicit different biological consequences.

Hence, in this section, we will summarize current approaches and applications of MGE analogs in modulating cell biological behaviors, including adhesion, differentiation, migration, homing, survival, and secretion, and the related mechanism is also summed up.

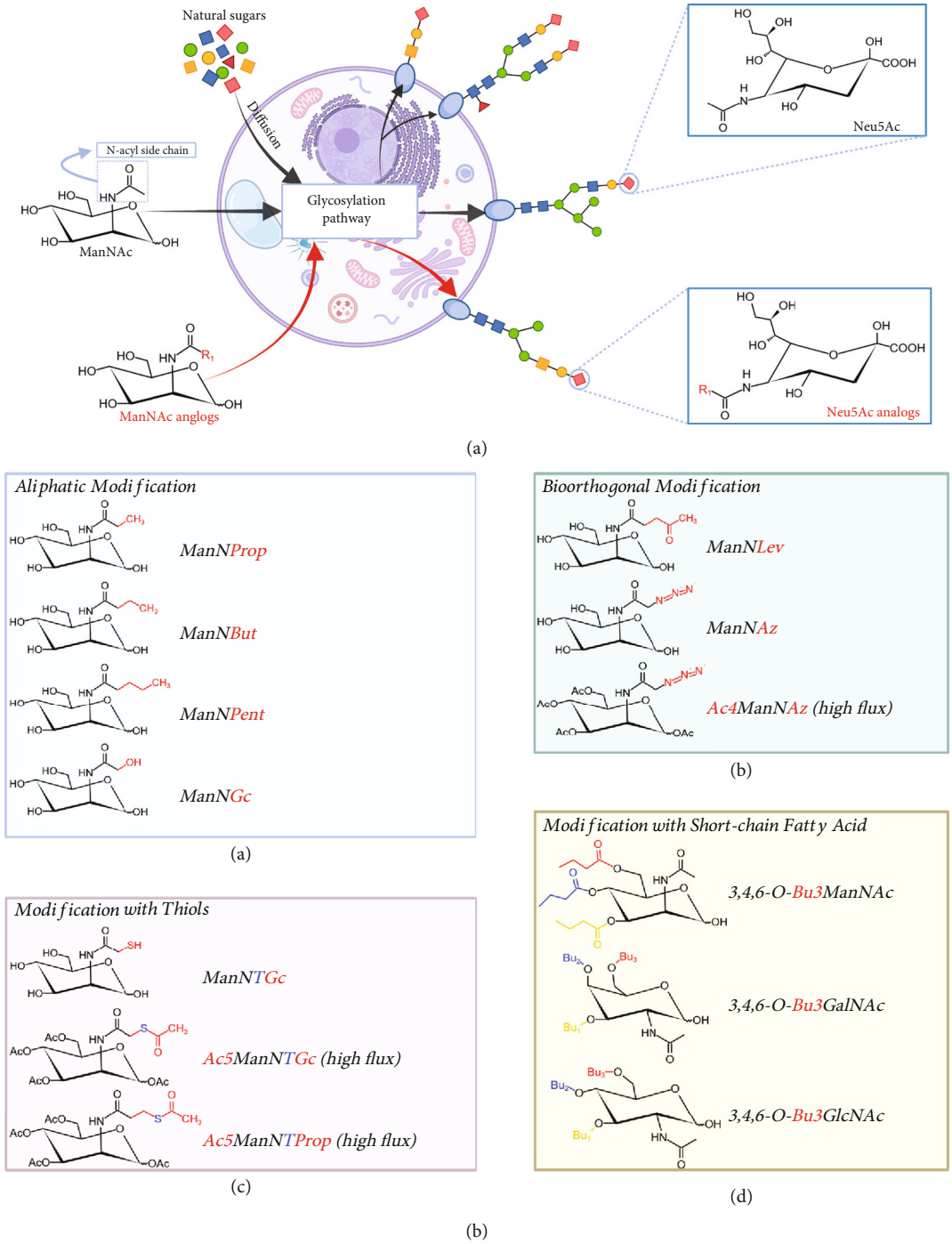

Figure 2: Overview of metabolic glycoengineering. (a) Illustration of MGE. Natural sugars are absorbed into cells and then metabolically assembled onto the cell surface as glycoconjugates (e.g., ManNAc into Neu5Ac). The analogs of metabolic precursors (ManNAc) can intercept the glycosylation pathways in cells, resulting in the submission of R-group-modified glycans and the modification of membranes. (b) Examples of MGE analogs. The analogs of MGE can be mainly classified into four catalogs: modification with aliphatic group, bioorthogonal group, thiol group, and short-chain fatty acid. The red marked text represents the R-group of different MGE analogs while the "high flux" indicates peracylated analogs with higher efficiency of modification.

4.1. Adhesion. Different chemical functional groups assembled on the surface can induce cell adhesion, and the strongest effect comes from methylene [75]. In 2002, Villavicencio-Lorini et al. reported that ManNProp stimulated the upregulation of intracellular β1-integrin receptors and eventually resulted in stronger adhesion between fibronectin and HL60 cells (the leukemia cell line) [76]. Although both natural and unnatural precursors of sialic acid can induce the upregulation of β1-integrins, the expression level induced by ManNProp (nonnatural) was twice than that of ManNAc (natural).

For the lack of the key enzyme of sialic acid pathway, HL60 cells do not express any sialylated molecules under normal conditions. But by incorporating Neu5Prop (Figure 2(a)) onto the glycans of HL60 cells, ManNProp dramatically increased the sialyl Lewis X (sLeX) biosynthesis, resulting in the promoted adhesion between HL60 cells and selectins [52]. The same effect of sLeX increasing was also detected in ManNProp-treated mesenchymal stromal cells (MSCs), which have been shown to target MSCs to bone marrow [77, 78].

Furthermore, the half-life time of some glycoproteins is modulated by terminal sialic acid. Thus, by altering the metabolic flux of sialic acid, MGE not only changes the glycan structure on the cell surface but can also affect the biostability of certain proteins. For example, ManNProp treatment extended the half-life of the sialylated molecule (CEACAM1) from 26 hours to 40 hours, which mediates cell-cell adhesion in PC12 cells (the rat pheochromocytoma cell line) [79].

Apart from methylene (-CH3), thiol is another kind of functionality that can promote cell adhesion.

Ac5ManNTGc is the hyperacetylated ManNAc analog with a thiol group on its N-acyl side chain (Figure 2(b)Bc). Jurkat cell is a T-lymphoma-derived cell line which possesses no adhesive property, but Ac5ManNTGc can incorporate thiols into the nonadhesive Jurkat cells [80] and then stimulate the additional cell adhesion to maleimide-functionalized surfaces [81]. These modified Jurkat cells also clustered spontaneously to produce numerous ECM components and upregulate their expression of β1-integrin, MMP-9, and CD44, which are involved in their attachment to ECM during T-lymphoma metastasis [82].

In these seminal explorations above, researchers generally use aliphatic analogs to modify membrane and elicit direct cell adhesion. But recently, bioorthogonal analogs (Figure 2(b)Bb) are also being exploited to enhance artificial adhesion since the click chemistry could endow cells to be chemically connected, building the "functional cell complexes." For example, Ac4ManNAz introduces azide groups into the cell surface as the first step; secondly, those azide groups are modified, respectively, with tetrazine (Tz) or *trans*-cyclooctene (TCO); thirdly, by mixing the modified cells, Tz-TCO click chemistry could produce intercellular adhesion [83, 84]. Click reaction between cyclodextrins (CDs) and adamantly (Adam) was also used to artificially combine A459 lung tumor cells and Jurkat cells, then triggered the activation of NK cells, and leads to the death of cancer cells [85].

In summary, the adhesion of cells to their microenvironment is indispensable because a cell cannot survive in the manner of an individual [3]. Because sialic acid participates in cell-cell and cell-matrix adhesion, it is worth wondering whether MGE can decide cell fate by introducing nonnatural glycans.

4.2. Differentiation and Proliferation. The earliest application of metabolic glycoengineering in regenerative medicine was conducted in neural cells by Schmidt et al. in 1998. ManNProp incubation in neural progenitor cells (NPC) induced the proliferation of astrocytes, microglia, and early-stage oligodendrocytes [86]. Buttner et al. also reported that ManNProp stimulated axonal outgrowth both in neuron cells and PC12 cells [87].

It has been demonstrated that ManNProp increased the calcium fluctuation in these cells [88]. Likewise, the adhesion effect of ManNProp in HL60 cells was also caused by intracellular calcium spiking, which thereby promoted the cell marker of monocytic differentiation [89]. And systemic administration of ManNProp significantly increased the axonal regeneration after sciatic nerves were transplanted into the mouse model [90], which proved to depend on the polysialyltransferase activity in the nerve graft [91].

It was suggested for the first time that ManNAc analogs can directly influence proliferation and axonal growth, and it is worth exploring for their influence on differentiation.

As described in Section 4.1, Ac5ManNTGc endows Jurkat cells with adhesive properties by introducing thiols. Moreover, when being applied in the human embryoid body–derived (hEBD) stem cells, Ac5ManNTGc was proved to stimulate neural lineage differentiation in the absence of Wnt signaling proteins [81], which are usually indispensable for neural differentiation [92].

In contrast, those cells treated with Ac5ManNGc—-which lacks thiol—showed only slight changes in the cytoskeleton, indicating no influence on differentiation. It can be inferred that the thiol group of the ManNAc analog is the key factor in enhancing cell adhesion and neural differentiation. But the upregulation of Wnt signal was only found on a gold-covered surface, indicating that the high-affinity bond of thiols was confined to the complementary scaffolds.

Du et al.'s group recently synthesized two novel thiol analogs—Ac5ManNTProp and Ac5ManNTBut (Figure 2(b)Bc)—which are claimed to install thiol on an elongated N-acyl side chain, thereby enhancing the ability of glycans to interact with other thiols, and overcome the necessity for complementary scaffolds [93]. When treated with human neural stem cells (hNSCs) and human adipose-derived stem cells (hADSCs), respectively, the stronger morphological responses were observed with Ac5ManNTBut, while Ac5ManNTProp exhibited better biocompatibility.

Among the thiol-treated hNSCs, the apparent neural differentiation was found as well as the upregulation of the Wnt signaling pathway. It was also demonstrated that the longer the N-acyl side chains were, the stronger the activation of Wnt signaling would be—raising from Ac5ManNTGc to Ac5ManNTProp and the strongest up to Ac5ManNTBut. According to the fact that the Wnt signaling also hinders adipogenesis [94], both Ac5ManNTProp and Ac5ManNTBut did inhibit the adipocyte differentiation in hADSCs [93]. The biosafety and scaffold-independent properties will make those ManNAc analogs be attractive tools in neural regenerative medicine.

In the previous studies, thiol-modified ManNAc analogs were usually used as bioorthogonal handles to link drugs to antibodies through maleimide conjugation [95]. But the work of Yarema et al. demonstrated that bioorthogonal analogs may also induce direct biological responses similar to those aliphatic analogs. And the outcome of thiol

modification could be changed by altering the length of the aliphatic side chain.

By introducing topological cues in the growth substrate and creating the glycoengineered binding interface, cell adhesion can be enhanced, gene expression can be regulated, and thus cell fate can be controlled, where the chemical composition of the cell surface is altered to promote carbohydrate-mediated interaction.

4.3. Migration and Homing. Beyond differentiation, cell migration and homing are regarded as the other two important activities that associated with sialic acid.

For instance, tumor cells usually synthesize polysialic acid (polySia) to regulate their ECM adhesion and migrate properties thereby becoming more metastatic [96]. Nagasundaram et al. reported a remarkable reduction of polySia in MCF7 breast cancer cells when treated with a series of ManNAc analogs (ManNProp, ManNBut, and ManNPent). Furthermore, the decreased level of natural polySia significantly suppressed adhesion and then inhibited breast cancer migration [97].

Conversely, compared with cancer cells, stem cells showed opposite responses. Incubation of ManNProp endows MSCs with a high expression level of sLeX which enhanced their osteotropism, also known as "homing" [77]. This homing effect of MSCs is determined by the interaction between sLeX and selectins, which is also involved in the neurotropism of NSCs [98].

In addition, supplementation with 3F-Neu5Ac was proved to increase migration and adhesion of MSCs and then promoted their survival rate in an ischemia model [63]. And the inhibition of osteogenic and adipogenic differentiation was also observed in those MSCs treated with 3F-Neu5Ac.

4.4. Apoptosis, Survival, and Secretion. The fact that sialic acid metabolism participates in cell apoptosis [54, 99] is also reflected in ManNAc analogs. By modulating metabolic flux of the sialic acid pathway, ManNAc analogs have the potential to either amplify or reduce cell apoptosis.

Research by Kim et al. demonstrated that ManNAc analogs can modulate cell apoptosis directly through N-acyl group effects, or indirectly via hydroxyl group effects. Especially, the ketone-bearing analog (Ac4ManNLev) possesses strong toxicity via inhibiting the sialic acid pathway [100]. Furthermore, the combination of 3,4,6-O-tributanoylation with the ManNLev (3,4,6-O-Bu3ManNLev) resulted in the most apoptotic ManNAc analog, which thus being a promising anticancer drug candidate [101]. Instead, 1,3,4-O-Bu3ManNAc increased the sialylation level of SW1990 cells (pancreatic cancer cells) up to 2-fold and in essence resensitize the SW1990 cells to anticancer drugs [102].

While the ketone groups are used to induce cell apoptosis, azide groups are promising in enhancing the survival rate of those cells seeded in biomaterials. Mao et al. fabricated DBCO-modified polymers as an MGE responsive platform and obtained azide-labeled macrophages through Ac4ManNAz. The bioorthogonal reaction between DBCO and azide accomplished fast in situ cellularizations and dramatically increased the selective capture and survival rate of the macrophages in the DBCO-modified scaffold [103].

Another intriguing application is the single-cell encapsulation via MGE. Oh et al. [104] utilized DBCO-azide conjugation to wrap every neural progenitor cell (NPC) with a layer of PEG polymer. The single-cell encapsulation with optimized stiffness changed the ADCY8-cAMP pathway due to the mechanical properties of polymers and enhanced the trophic factor secretion of NPCs, which reduced the required amounts of cells for therapy.

4.5. Brief Summary. All in all, the sialylation of N-glycans is tightly associated with the response to microenvironmental cues. Altering the structure of cell-surface glycans by metabolic glycoengineering (MGE) will certainly affect signaling pathways, no matter of incorporating either bioorthogonal or aliphatic modification.

From the analysis of the microarray data, up to a total of 14 pathways have been proved to be modulated via MGE products [105], including apoptosis, cell adhesion molecules, cell differentiation, leukocyte migration, and Wnt signaling as well as NF-κB signaling.

Taken together, these applications have established the MGE analogs as versatile tools for modulating biological activity such as cell adhesion, differentiation, migration, survival, or secretion, which may positively impact the therapeutic potential of the stem cells.

5. Applying MGE in Regenerative Medicine: With a Focus on Musculoskeletal System

Sialic acid possesses another name called "neuraminic acid," since it was initially isolated from neural tissues and highly expressed in the neural system. Hence, the earliest therapeutic exploration of MGE had mostly focused on neural lineage differentiation and neural tissue regeneration.

But sialic acid is widely existing in plentiful cells and tissues, rather than exclusively in neural systems. Along with the prospering development of MGE, this carbohydrate-based strategy also showed potential in therapy for the musculoskeletal system. In this section, we will introduce the application of MGE in regenerative medicine with a focus on the intervertebral disc and cartilage, which all tend to be ideal targets for metabolic glycoengineering.

5.1. Chondrogenic Differentiation and Cartilage Tissue Regeneration. The inflammatory environment in osteoarthritis (OA) joints disrupts the homogenesis of the articular microenvironment, thus reducing the ability of cartilage to regenerate and limiting the efficacy of OA therapeutics. To date, some carbohydrate-based molecules have shown potential in stem cell differentiation and chondrocyte regeneration.

5.1.1. Glucosamine-Metabolic Glycoengineering Produced by "Nature." Glucosamine (GlcN), a natural sugar that widely exists in cartilage, serves as the precursor for glycosaminoglycans (GAG), which are important components of the ECM secreted by chondrocytes and help sustain the

flexibility, toughness, and strength of this connective tissues [106]. Probably, we could define that glucosamine is kind of like a natural precursor of MGE since it helps with the supplement of ECM as many other analogs did.

It is well-known that GlcN has been regarded as a proper chondroprotective drug candidate for decades [107]. In 2007, Derfoul et al. demonstrated that the treatment of GlcN contributed to maintaining the chondrogenic phenotypes both in osteoarthritic chondrocytes and MSCs and promoted the secretion of ECM, as well as partially inhibited the expression of IL-1β and matrix metalloproteinase-13 (MMP-13) [108], which account for the clinical therapeutic effect of GlcN on OA, such as anti-inflammatory and chondroprotective.

In 2005, Khoo et al. reported the inducing effect of GlcN on the differentiation of embryonic stem cells (ESCs) [109]. By encapsulating GlcN in hydrogels, it was demonstrated that GlcN significantly enhanced the accumulation of chondrogenic ECM in the embryonic body (EB) [110]. Moreover, recent studies have shown that the treatment of GlcN also promoted the proliferation of chondrocytes via the Wnt/β-catenin signaling pathway [111], similar to the stem cells treated with ManNAc analogs [93].

The N-butyryl analog of glucosamine, "GlcNBut," was also found to stimulate normal chondrocytes to secrete ECM [112]. Poustie et al. cultured the normal chondrocytes with several glucosamine analogs, including GlcNAc, GlcNBut, and GlcNProp. The treatment of GlcNBut to chondrocytes increased the level of expression of mRNA of collagen-II and aggrecan, while GlcNAc and GlcNProp had no such influences. GlcNBut showed its potential to alleviate OA disease, but whether it could be applied *in vivo* to rejuvenize the senescent cartilage remained uncertain.

5.1.2. Tributanoylated GlcNAc, GalNAc, and ManNAc: The Hexosamine Analogs Derived from Short-Chain Fatty Acid. Despite the widely reported anti-inflammatory and chondrogenic differentiation properties of GlcN, its direct influence on the regeneration of cartilage had remained inconclusive for a long time [113, 114]. From a perspective of tissue engineering, the effective treatment of osteoarthritis requires a strategy that can both reduce inflammation and increase tissue production.

Among the efforts in reducing inflammation, NF-κB signaling is an intriguing therapeutic target in OA disease since it regulates the expression of many inflammatory mediators and matrix-degrading enzymes [115].

As it is mentioned in Section 4.5, the NF-κB signaling pathway can be modulated via MGE products [105], and the inhibition of NF-κB in cancer cells was previously observed with the short-chain fatty acid- (SCFA-) modified hexosamine analogs (Figure 2(b)Bd), such as tributanoylated 3,4,6-O-Bu3ManNAc and 3,4,6-O-Bu3ManNLev [101, 116]. After cellular uptake, those tributanoylated sugars can be naturally metabolized to their downstream byproducts, for example, from 3,4,6-O-Bu3ManNAc to ManNAc with three butyrate groups. The hexosamine part acted as a "core" for the biosynthesis of glycosaminoglycans (GAG), while the butyrate moieties modulated inflammation signaling pathways.

In addition to 3,4,6-O-Bu3ManNAc, the tributanoylated GlcNAc analog (3,4,6-O-Bu3GlcNAc) downregulated the NF-κB activity in those cancer studies too [116], which was reminiscent of the therapeutic effect of GlcNAc analogs in OA. And not surprisingly, the same effect of the tributanoylated GlcNAc (3,4,6-O-Bu3GlcNAc) was observed in OA chondrocytes, which promoted their ECM accumulation and inhibited inflammation [117], indicating that 3,4,6-O-Bu3GlcNAc might have the potential to reproduce cartilage tissue.

To get a comprehensive grasp of the characteristic of tributanoylated hexosamine analogs and to optimize their therapeutic effect in OA, Coburn et al. synthesized three analogs named as 3,4,6-O-Bu3GalNAc, 3,4,6-O-Bu3ManNAc, and 3,4,6-O-Bu3GlcNAc, for evaluating their effect on chondrocytes and mesenchymal stem cells, [118]. All the analogs inhibited the expression of NF-κB and increased the cartilage-like ECM accumulation in OA chondrocytes, while the GalNAc-Bu3 induced the strongest responses at a concentration with negligible cytotoxicity.

The *in vivo* investigation of GalNAc-Bu3 in rat OA models by Kim et al. showed that GalNAc-Bu3 induced cartilage tissue production both in MSCs and human OA chondrocytes by regulating the Wnt/β-catenin signaling, the negative pathway engaged in OA same as NF-κB [119]. Furthermore, GalNAc-Bu3 extended the survival of MSCs despite the rapid clearance rate of the synovial fluid.

Notably, the therapeutic effects of these tributanoylated analogs are not simply due to their catabolized metabolites, since their isomers are incapable to suppress the NF-κB activity (such as 1,3,4-O-Bu3GlcNAc), suggesting that the specific location of butyrate influences those effects [117].

Taken together, the potential of hexosamine analogs derived from SCFA could be translated as suitable drug candidates for OA disease, and the prospect of MGE-based carbohydrates in inducing chondrogenic differentiation of MSCs is calling for further investigations.

5.2. Prospect of MGE in Intervertebral Disc Regeneration. The intervertebral disc (IVD) is comprised of nucleus pulposus (NP), annulus fibrosus (AF), and endplates (EPs), while the NP is the highly hydrated region that is located at the inner central part of IVD [120]. It is well-known that the aging and dysfunction of NP cells lead to the degeneration of NP, which is an important initial process in the pathology of intervertebral disc degeneration (IVDD) [121].

5.2.1. Values and Obstacles of Stem Cells in IVDD Regeneration. Exogenous supplementation of mesenchymal stem cells (MSCs) has been proved to enhance the height and water content of the intervertebral disc [122, 123]. However, the harsh microenvironment in the degenerated NP (hypoxia, lack of blood supply, acidity, and hyperosmolality) severely hinders the survival and function of any transplanted cells [124–126].

As has been discussed earlier, the effective adhesion between stem cells and ECM can optimize cell behavior and tissue regeneration [127]. In the context of discs, it has been reported that the high affinity between MSCs and type

II collagen promoted the differentiation towards NP cells and helped MSCs maintain NP-like phenotypes [128, 129]. But a proper method to ensure the adhesion between transplanted cells and ECM components *in vivo* remains unclear in IVDD therapy. Herein, the MGE technique might help.

5.2.2. Enhance Nucleus Pulposus Regeneration by MGE. Based on previous studies demonstrating that modification of cell-surface glycan can modulate the adhesion property of cells [89], MGE might stimulate inner bounding to ECM that facilitates the differentiation of MSCs and then regenerate the disc.

An example from our laboratory validated that ManNProp, introducing extra -CH3 on sialic acid, greatly enhanced the adhesion ability of ADSCs, especially the selective adhesion with type II collagen [130]. Moreover, this cellular response promoted the efficiency of differentiation towards NP-like cells in a β1-integrin-dependent manner, by stimulating FAK/ERK pathway.

In rat IVDD models, better mechanical performance, increased water content, and the reconstruction of NP structure were observed after the transplantation of engineered cells with collagen scaffolds. It manifested that the MGE strategy not only promoted stem cells to overcome the harsh microenvironment in degenerated NP tissues but also benefits the regeneration of NP.

Designing biomaterials to improve cell adhesion is not a novel topic. However, the current efforts are mainly focusing on ECM engineering to "lure" passive adhesion by adding purified proteins and molecules [131, 132]. But the complexity of the synthetic procedure and the instability of proteins have confined the translational research of ECM modification. To address these limitations, we have highlighted the feasibility of cell membrane modification to produce "active adhesion" via glycoengineering and thus benefit disc regeneration.

Meanwhile, cartilage is an analogous connective tissue of IVD with a similar biofunction. And the type II collagen scaffolds carrying stem cells have also been exploited for cartilage defects [133]. Consequently, we assume that the MGE based on ManNProp is also potential in cartilage repair.

6. Outlook and Conclusion

Metabolic glycoengineering (MGE), a technique that prospered for three decades, is now shedding new light on extensive fields. In this review, we highlighted the determinable role of glycan in regulating cell microenvironment and analyzed how MGE modifies these glycans to regulate cell fate.

Beyond those biological activities discussed in this manuscript, the MGE strategy has also been applied in a variety of biomedical fields, such as visualizing glycoconjugates for *in vivo* tracking [134], targeting agents to diagnose or kill cancer cells [135], homing of therapeutic cells [136, 137], drug delivery to promote disease recovering [138], and cell vaccine-based immunotherapies [139]. Notably, to realize the full potential of MGE, it will be necessary to explore its unveiled opportunities in regenerative medicine and bridge the gap in the current research.

In the future, we can assume the direction of MGE as (1) extend MGE application to other glycosylation pathways beyond sialic acid and discover novel metabolic precursors; (2) determine the effect of MGE in more disease models and cell types that require better regenerative distribute, such as myocardial infarction (cardiomyocytes), diabetes (pancreatic beta cells), leukemia (hematopoietic stem cells), or any other disease suitable for stem cell transplantation; (3) bioorthogonal modification of stem cells holds great prospect since the click chemistry allows more complex combination, which may lead to broader methods in altering the stem cell niche; (4) the sustainability of modified groups due to natural catabolism of glycans ought to be ameliorated; and (5) translating the current development of MGE into clinical practice has to be on its way.

In conclusion, this review summarized the MGE's application in tissue engineering and regenerative medicine, and the booming diversity of MGE ensures a broad prospect for this technique in the future.

Data Availability

Data sharing is not applicable to this article as no new data was created or analyzed in this study.

Conflicts of Interest

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

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Research Article

Adipose-Derived Stem Cells Exosomes Improve Fat Graft Survival by Promoting Prolipogenetic Abilities through Wnt/β-Catenin Pathway

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Autologous fat grafting has been widely used in plastic surgery in recent years, but the unstable retention of fat graft has always been a key clinical problem. Adipose tissue has poor tolerant to ischemia, so the transplanted adipose tissue needs to rebuild blood supply at an early stage in order to survive stably. Our previous study has found that comparing to human foreskin fibroblast exosome (HFF-Exo), human adipose-derived stem cells exosome (hADSC-Exo) can significantly improve the proliferation of vascular endothelial cells and the angiogenic effect of artificial dermal preconstructed flaps. Therefore, the ability of hADSC-Exo to improve the retention of adipose grafts and its potential regenerative mechanism aroused our strong interest. In this study, we applied hADSC-Exo and HFF-Exo to adipose grafts and explored the potential regeneration mechanism through various means such as bioinformatics, immunofluorescence, immunohistochemistry, and adipogenic differentiation. The results showed that hADSC-Exo can significantly promote grafts angiogenesis and adipogenic differentiation of ADSC to improve the retention of fat grafts and may downregulate the Wnt/β-catenin signaling pathway to promote the adipogenic differentiation. In summary, our results provide a theoretical basis for the clinical translation of hADSC-Exo in fat grafting.

1. Introduction

Autologous fat grafting is widely used for various treatments in plastic surgery, such as depressed scarring, hemifacial atrophy, and facial rejuvenation, due to its good biocompatibility, low tissue rejection rate, and allergic reaction rate [1, 2]. However, the unstable retention of fat grafts has been a key clinical problem to be overcome. Adipose tissue has poor tolerant to ischemia, liposuction will result in the destruction of their original blood supply, and the transplanted adipose tissue needs to rebuild blood supply at an early stage in order to survive stably [3]. Yoshimura et al. [4] found that grafted fat mostly showed hematologic reconstruction only in the peripheral area, while the central area showed fat necrosis and resorption due to insufficient blood supply and a decrease in grafted tissue volume. Therefore, enhancing hematologic reconstruction in the early stages of fat grafting is one of the key factors to promote graft survival.

Adipose-derived stem cell (ADSC) is a type of stem cell derived from adipose tissue with multidirectional differentiation potential, which is widely used in the field of regenerative medicine in recent years because of its easy extraction and expansion and strong tissue repair activity [5]. Numerous studies [6, 7] have shown that cotransplantation of autologous adipose tissue with ADSC, which are capable of

paracrine secretion of various cytokines to promote early vascularization, significantly improves the survival rate of adipose grafts. Interestingly, our previous study [8] found that ADSC can differentiate into vascular endothelial cells and enhance the proliferation and migration of vascular endothelial cells to promote the healing of chronic wounds. However, the tumorigenicity of ADSC and the storage and transport of cellular products are still controversial, and there is a lack of uniform standards for the application of ADSC products [9, 10]. Thus, ADSC does not have the conditions for clinical large-scale application.

Exosomes are a class secreted by cells into the extracellular matrix that can carry a variety of biological components such as proteins, lipids, and noncoding RNAs and can be transported to specific target cells or bound to the cell surface to mediate intercellular communication [11, 12]. ADSC-derived exosome (ADSC-Exo) has been used in ischemic injury diseases and has shown good vascular regenerative effects similar to ADSC, and has the advantages of no risk of tumor formation, high stability, and easy storage and transport, making it more promising for clinical application than ADSC-based cell therapies [13]. In our previous study [14], hADSC-Exo significantly promoted the proliferation of vascular endothelial cells and the angiogenic effect of artificial dermal preconstructed flaps compared to human foreskin fibroblast exosome (HFF-Exo). Therefore, the ability of hADSC-Exo to improve the retention of adipose grafts and its potential regenerative mechanism aroused our strong interest.

We therefore applied hADSC-Exo and HFF-Exo to adipose grafts in this study and found that hADSC-Exo were capable of significant graft vascular regeneration and adipogenic differentiation of ADSC to improve the retention of adipose grafts. Meanwhile, bioinformatics technology was used to explore the potential mechanisms of hADSC-Exo that promote the adipogenic differentiation of stem cells.

2. Material and Method

2.1. Identification of hADSC. The fourth passage of hADSC was selected for flow cytometry analysis for phenotypic identification of hADSC surface markers. CD90 and CD34 (BD Biosciences, San Jose, CA, USA) were used for flow cytometry analysis. We reported the staining steps previously [8].

2.2. Isolation of Exosome. Human adipose-derived stem cells (hADSC) with passages of 3-6 and human foreskin fibroblast (HFF) with passages of 3-6 were cultured for obtaining hADSC-Exo and HFF-Exo. The hADSC and HFF were cultured in low-glucose and high-glucose DMEM (HyClone, Utah, USA), respectively, and supplemented with 10% exosome-free fetal bovine serum (FBS) (Gibco, Grand Island, USA) and incubated with 5% CO_2 at 37°C. Exosomes were isolated from hADSC and HFF culture medium by differential ultracentrifugation as previously described [15]. In brief, differential ultracentrifugation at 300×*g* and 2000×*g* for 10 min, followed by 10,000×*g* for 30 min, followed by 100,000 × g for 70 min twice were all performed at 4°C. The exosome pellets were finally resuspended in 100 μL of phosphate-buffered saline (PBS) and filtered with 0.22 sterile filter before use.

2.3. Verification of Exosome

2.3.1. Transmission Electron Microscopy (TEM). Exosomes were imaged by TEM to identify their morphology. The exosome sample with a volume of 20 μL was prepared and dropped on a copper mesh for 10 min and stained with 20 μL of 3% sodium phosphotungstate solution for 5 min. Then, copper mesh was dried at room temperature for 30 min. Finally, the sample was detected by TEM.

2.3.2. Nanoparticle Tracking Analysis (NTA). Particle size distribution of exosomes was identified by NTA (ZetaView, Particle Metrix, Germany). The exosome sample is diluted to the appropriate concentration using PBS and detected on the NTA.

2.3.3. Western Blotting. The western blotting instruction was described previously [8]. In brief, the proteins from each sample were extracted and transferred to a PVDF membrane (Cell Signaling Technology, MA, USA). The membrane was initially blocked in 5% bovine serum albumin for 2 h and then incubated with primary CD63 (1 : 2000, Abcam, Cambridge, MA, UK), CD81 (1 : 1000, Cell Signaling Technology, USA), α-tubulin (1 : 1000, Cell Signaling Technology, MA, USA), VEGF-A antibody (1 : 1000, Abcam, Cambridge, MA, UK), and β-catenin (1 : 1000, Cell Signaling Technology, USA) at 4°C overnight, followed incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1 : 3000, Beyotime, China). An Alpha Imager scanner (Tecan, Thermo Fisher Scientific, USA) was used to visualize the protein expression by chemiluminescence.

2.4. Fat Grafting In Vivo. A total of nine nude mice (6 weeks old, male) were purchased from the experimental animal center of Navy Military Medical University (Shanghai, China). All experiments were approved by the guidelines of the Health Sciences Animal Policy and Welfare Committee of Navy Military Medical University.

Transplanted fat from human subcutaneous adipose tissue samples were obtained from the abdominal liposuction of a healthy women in the Changhai Hospital affiliated with the Navy Military Medical University and were obtained with informed consent of the patient. Adipose tissue (0.5 mL) added with hADSC-Exo or HFF-Exo (100 μg) was subcutaneously injected in two recipient sites on the dorsal surface. The transplanted fat was observed at 1, 2, and 3 months postoperatively. The weight and volume of survive fat were assessed.

2.5. Immunohistochemistry and Immunofluorescence. Adipose tissues were excised at 1, 2, and 3 months after injection and analyzed by histological staining. The transplanted fat tissues were fixed with 4% polymethylene formaldehyde and embedded with paraffin. Hematoxylin and eosin were used for histological observation of the intrinsic structure of the grafted fat [16]. Transplanted fat angiogenesis and proliferative cells were observed by $CD31^+$ (1 : 50, Abcam,

UK) and Ki67 (1 : 500, Abcam, UK) immunohistochemical staining, respectively. Differentiated fat cells were observed by PPARγ (1 : 250, Abcam, UK) immunofluorescence staining. The immunohistochemical and immunofluorescence assays were performed as reported previously [8]. Immunohistochemical photomicrographs were obtained under a Pannoramic DESK (3D HISTECH, Hungary) and visualized by Pannoramic scanner, and immunofluorescence photomicrographs were obtained with a Zeiss fluorescence microscope (HLA100, Shanghai, China).

2.6. Cell Proliferation Assay. Cell proliferation rates were tested using a Cell Counting Kit-8 (CCK-8) assay (Beyotime, Jiangsu, China) and a 5-ethynyl-2 deoxyuridine (EdU) assay kit (RiboBio, Guangzhou, China). HUVECs (2000 cells/well) were seeded in a 96-well plate and incubated with different concentrations of hADSC-Exo (50, 100 or 200 μg/mL) or equivalent amount of PBS. Cell proliferation was analyzed at 24 h, 48 h, and 72 h after treatment. A 10 μL CCK8 reagent was added to each well, and the cells were incubated for 2 h at each time point. Then, OD value was verified by a microplate reader (Tecan, Thermo Scientific, USA) at 450 nm. The three independent experiments were performed. Based on the CCK8 data, we determined a suitable hADSC-Exo concentration for the follow-up experiment.

An EdU assay kit was performed to compare the proliferative effects hADSC-Exo and HFF-Exo on HUVEC. HUVECs (5×10^4 cells/well) were seeded in a 96-well plate and cultured to reach 70%–80% confluency. Then, HUVECs were cocultured with the suitable hADSC-Exo concentrations and equal concentration of HFF-Exo for 72 h, and an EdU assay was performed according to the manufacturer's instructions [17]. Proliferating HUVECs were stained with green fluorescent, and all cell nuclei were stained with blue fluorescent and observed with the Zeiss fluorescence microscope.

2.7. Cell Migration Assay. Transwell assays were performed to compare the migration effects hADSC-Exo and HFF-Exo on HUVEC. HUVECs (5×10^4 cells, 200 μL) resuspended in FBS-free medium and added to the Transwell insert, and the well was added with medium containing 5% exosome-free FBS and hADSC-Exo/HFF-Exo (800 μL). After culture for 18 h, HUVECs were fixed and stained with 0.1% crystalline violet, and the cells that did not cross the membrane were carefully wiped to remove and imaged. The migration cells were measured by using ImageJ software (Bethesda, MD, USA).

2.8. Adipogenic Differentiation Assay. The hADSCs (5×10^5 cells) were seeded in 12-well plates and cultured with medium containing 5% exosome-free FBS added with hADSC-Exo or HFF-Exo until their confluency was 70-80%. Then, the cells were cultured with adipogenic differentiation medium (Cyagen, Guangzhou, China) as the manufacturer's instructions for 4 weeks [8]. Then, the cells were fixed and stained with oil red O. The stained cells were washed with PBS to away excess dye and imaged.

2.9. Differential Analysis of Gene Expression about Adipogenic Differentiation. The gene expression microarray dataset of hADSC during in vitro adipogenic differentiation (GSE61302), comprised of hADSC samples from 7-day adipogenic differentiation ($n = 5$), 21-day adipogenic differentiation ($n = 6$), and undifferentiated ($n = 5$), was downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo). The dataset was based on the GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array), and the mRNA expression data of 21-day adipogenic differentiated hADSC and undifferentiated control were downloaded and used for this study. The differential analysis of gene expression in late-stage adipogenic-differentiated hADSCs compared with undifferentiated cells was performed using the GEO2R analysis tool. An mRNA with a p value of <0.05 and a log FC value greater than ±1 was defined as a DEG, and the lists of upregulated and downregulated DEGs were collated for subsequent analysis.

2.10. Integration Analysis of Exosomes Contributing to Adipogenic Differentiation. The potential upregulated and downregulated target DEGs were selected, and the protein–protein interaction (PPI) networks were analyzed with the STRING database (https://string- http://db.org/). The hub genes in PPI networks were calculated by using the degree analysis method in the CytoHubba plug-in, and visualized by Cytoscape (version 3.9.0) software [18]. The DAVID database (https://david.ncifcrf.gov/home.jsp) was used to understand the biological function of genes, including gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment [19]. GO annotation analysis comprised of molecular function (MF), biological process (BP), and cell component (CC).

2.11. Statistical Analysis. Statistical analysis was performed using SPSS 26.0 and described as mean ± SD. Student's t -test were used to examine statistical significance, and a p value of <0.05 was considered significant.

3. Results

3.1. Characterization of hADSC and Exosomes. To assess the surface marker phenotype of hADSC, the flow cytometry analysis was used to identify the purified hADSC. Approximately 99% of hADSCs were positive for CD90, but negative for CD34 (Figure 1(a)), which suggested that the cultured cells were ADSC. Exosome is a class of membranous vesicles about 30-150 nm in diameter extracted by various methods including ultracentrifugation, exclusion ultrafiltration, density gradient centrifugation, particle size separation, polymer precipitation, and immunoaffinity [15, 20]. In this study, the identification of exosomes was performed by TEM, NTA, and western blotting. TEM confirmed the typical morphology of the exosomes (Figure 1(b)), and NTA showed that the average diameter of hADSC-Exo was 139.5 ± 93.3 nm and HFF-Exo was 131.0 ± 52.2 nm (Figure 1(d)). Moreover, specific membrane protein markers CD63 and CD81 were

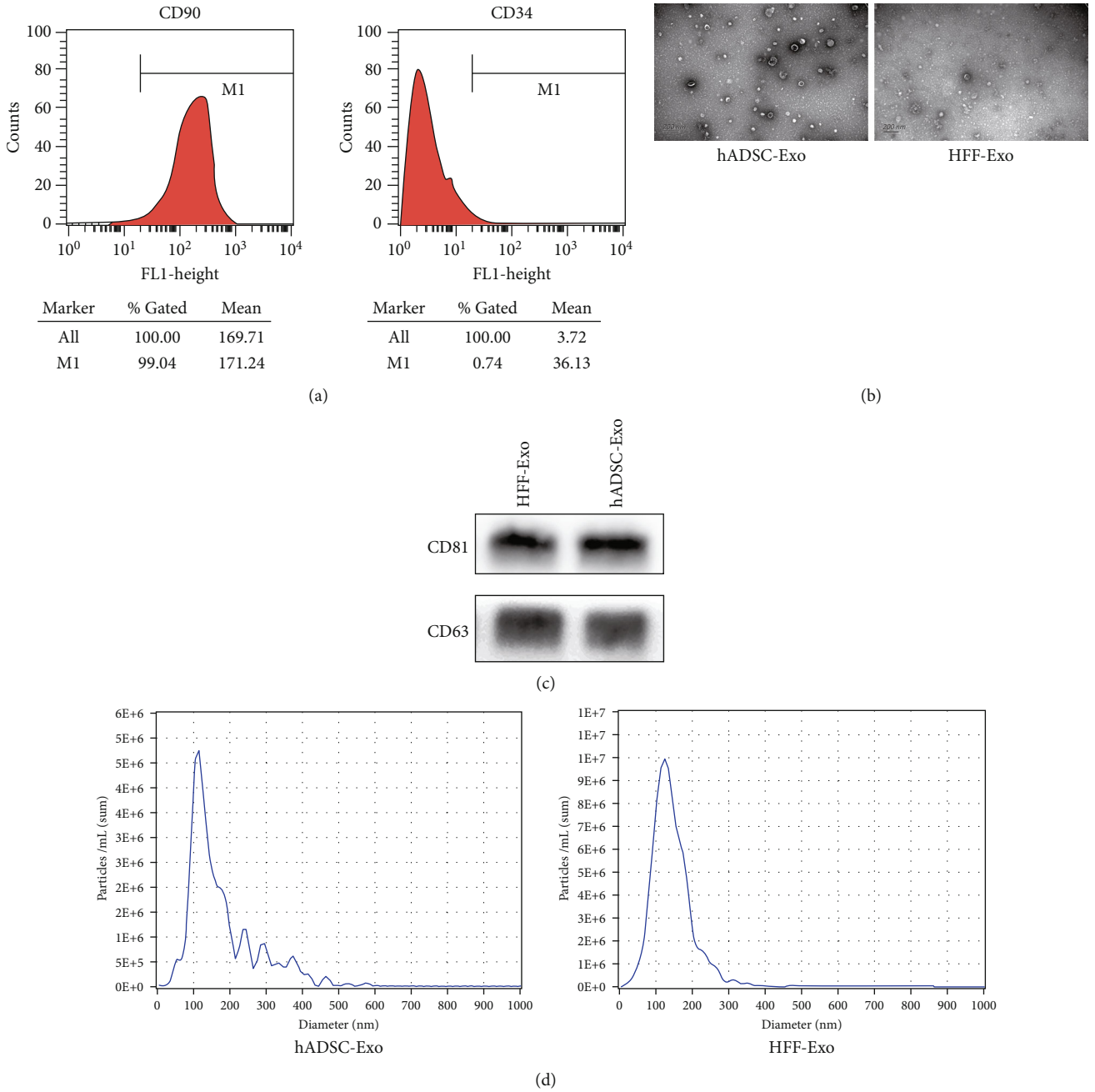

Figure 1: Identification of hADSC, hADSC-Exo, and HFF-Exo. (a) Flow cytometry analysis of cell surface marker CD90 and CD34 was showed in hADSC. (b) The image of exosome was showed through TEM. (c) Western blotting verified the expression of CD63 and CD81 in hADSC-Exo and HFF-Exo. (d) NTA measured the size distributions of hADSC-Exo and HFF-Exo.

also highly expressed in both exosomes (Figure 1(c)). These results were all consistent with the characteristics of exosomes.

3.2. hADSC-Exo via Proangiogenic and Prolipogenetic Effects Improves Fat Graft Survival In Vivo. To assess the retention beneficial of hADSC-Exo on adipose grafts, nude mice were used as fat grafting models (Figure 2(a)). The grafts were harvested at 1, 2, and 3 months after fat grafting. A general observation of fat specimens at three time points revealed that the hADSC-Exo had better retention efficiency than HFF-Exo (Figure 2(b)). Then, the weight and volume of grafts were measured and found that the sizes of grafts decreased with the transplantation time, but the hADSC-Exo had better quantitative results than HFF-Exo (Figures 2(c)–2(h)), indicating an excellent effect of hADSC-Exo on grafted fat survival.

For microscopic assessment of the effect of hADSC-Exo in promoting the retention of adipose grafts, HE staining results (Figures 3(a) and 3(b)) revealed that the transplanted fat from hADSC-Exo groups had higher adipose number and more complete adipose morphological structure

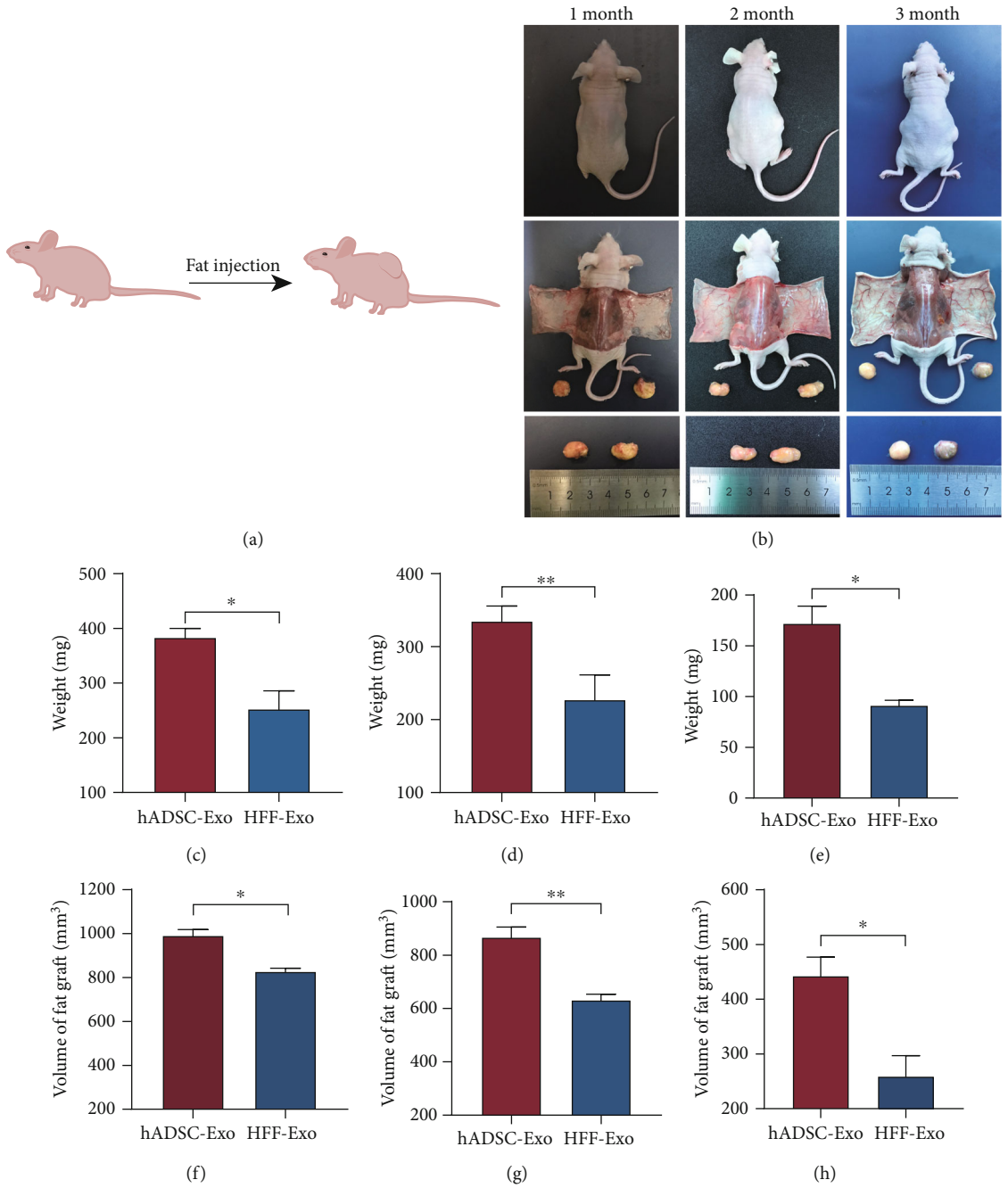

Figure 2: hADSC-Exo enhanced the survival rate of fat grafts in the nude mouse model. (a) The design of the nude mouse model. (b) Gross observation of the fat grafts demonstrated that the hADSC-Exo group (the right side of each picture) had larger graft sizes compared with that in the HFF-Exo group (the left side of each picture). (c–f) Harvesting the fat grafts at 1, 2, and 3 months after fat transplantation, the weight and volume of fat grafts were significantly better in the hADSC-Exo group compared to the HFF-Exo group ($*p < 0.05$, $**p < 0.01$, and $*** p < 0.001$).

compared to the HFF-Exo groups. The degree of vascular regeneration and the number of proliferating cells are also important indicators to evaluate the retention of fat grafts. $CD31^+$ and Ki67 immunohistochemistry staining were performed and observed that the number of CD31-positive blood vessels and Ki67-positive proliferating cells in the hADSC-Exo group were significantly higher than that in the HFF-Exo group (Figures 3(c)–3(f)).

3.3. Proangiogenic Effect of hADSC-Exo In Vitro. To examine the proangiogenic capacity of hADSC-Exo and HFF-Exo, HUVEC proliferation and migration assays were performed. We first examined the effect of hADSC-Exo on HUVEC proliferation using CCK8 cell proliferation assay to determine the optimal concentration of exosomes for subsequent experiments. The results showed that 100 μg/mL of hADSC-Exo had the most significant promotion effect on HUVEC

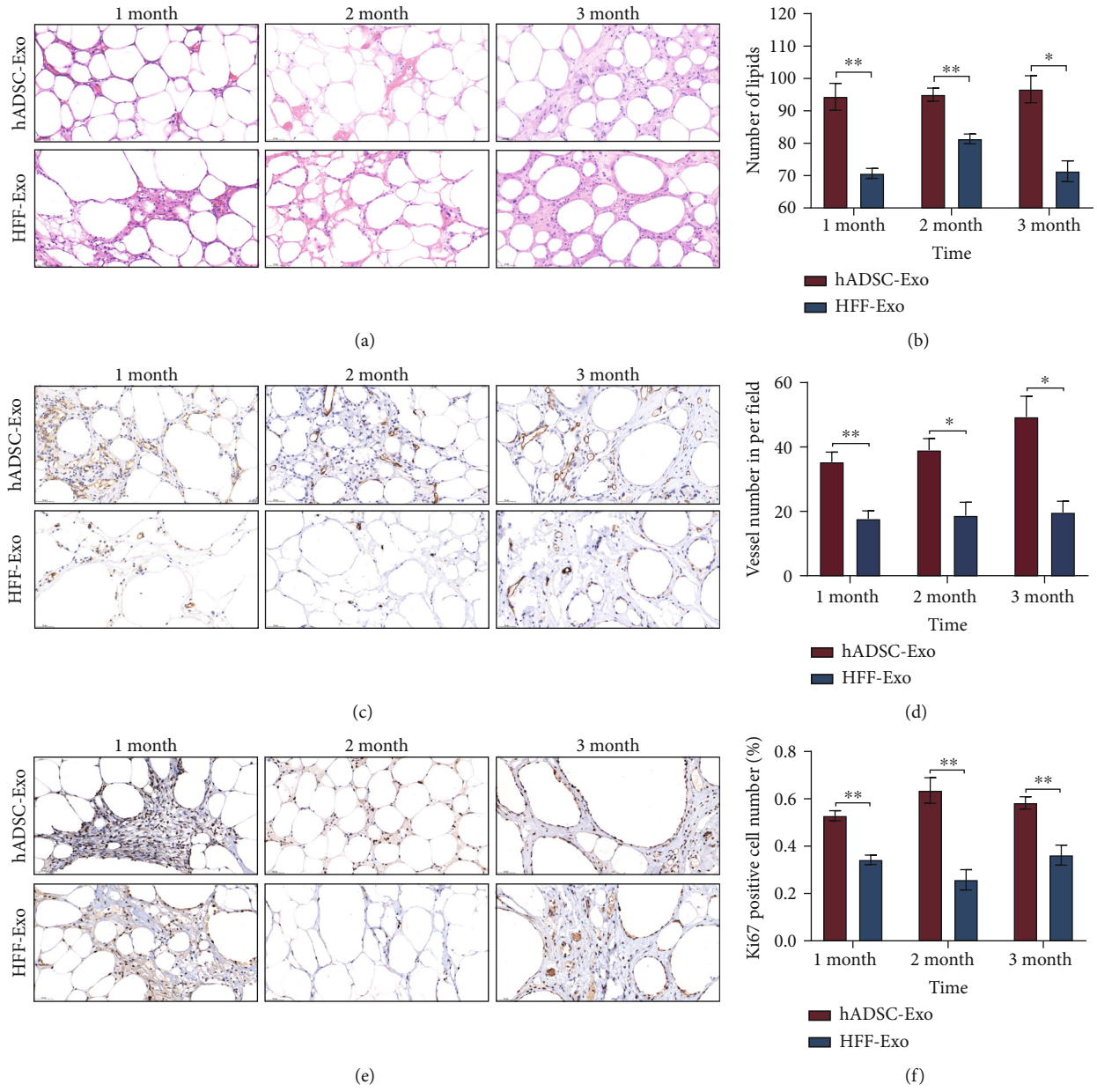

Figure 3: hADSC-Exo promoted the retention and neovascularization of adipose grafts in the nude mice fat grafting model. (a) HE staining revealed that the transplanted fat in the hADSC-Exo groups showed higher adipose number and more complete adipose morphological structure compared to the HFF-Exo group. (b) Semiquantitative scale of integrity of HE staining. (c–f) The number of CD31-positive blood vessels and Ki67-positive proliferating cells in the hADSC-Exo group were significantly higher than that in the HFF-Exo group ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

proliferation, and this promotion effect persisted with increasing culture time (Figure 4(a)). Therefore, we used 100 μg/mL as concentration of hADSC-Exo and HFF-Exo for subsequent experiments. EdU proliferation assays and Transwell assays were used to evaluate the effect of both exosomes on HUVEC proliferation and migration, respectively. Notably, hADSC-Exo were able to promote HUVEC proliferation and migration more effectively than HFF-Exo (Figures 4(d)–4(g)).

VEGFA was initially shown to be an endothelial growth factor and a regulator of vascular permeability [21] and is closely associated with angiogenesis in a variety of conditions, including tumors and wounds [21, 22]. Thus, the protein expression of VEGFA was evaluated and found that hADSC-Exo group exhibited a higher degree of expression (Figures 4(b) and 4(c)). Our finding indicated that hADSC-Exo significantly promoted angiogenesis in adipose grafts.

3.4. Identification of Key Target Genes for Lipogenesis by hADSC-Exo. After observing that the hADSC-Exo group had superior adipose number and structural integrity, whether hADSC-Exo could promote adipogenic differentiation of the original ADSC in adipose tissue has aroused our strong interest. Therefore, we performed the immunofluorescence

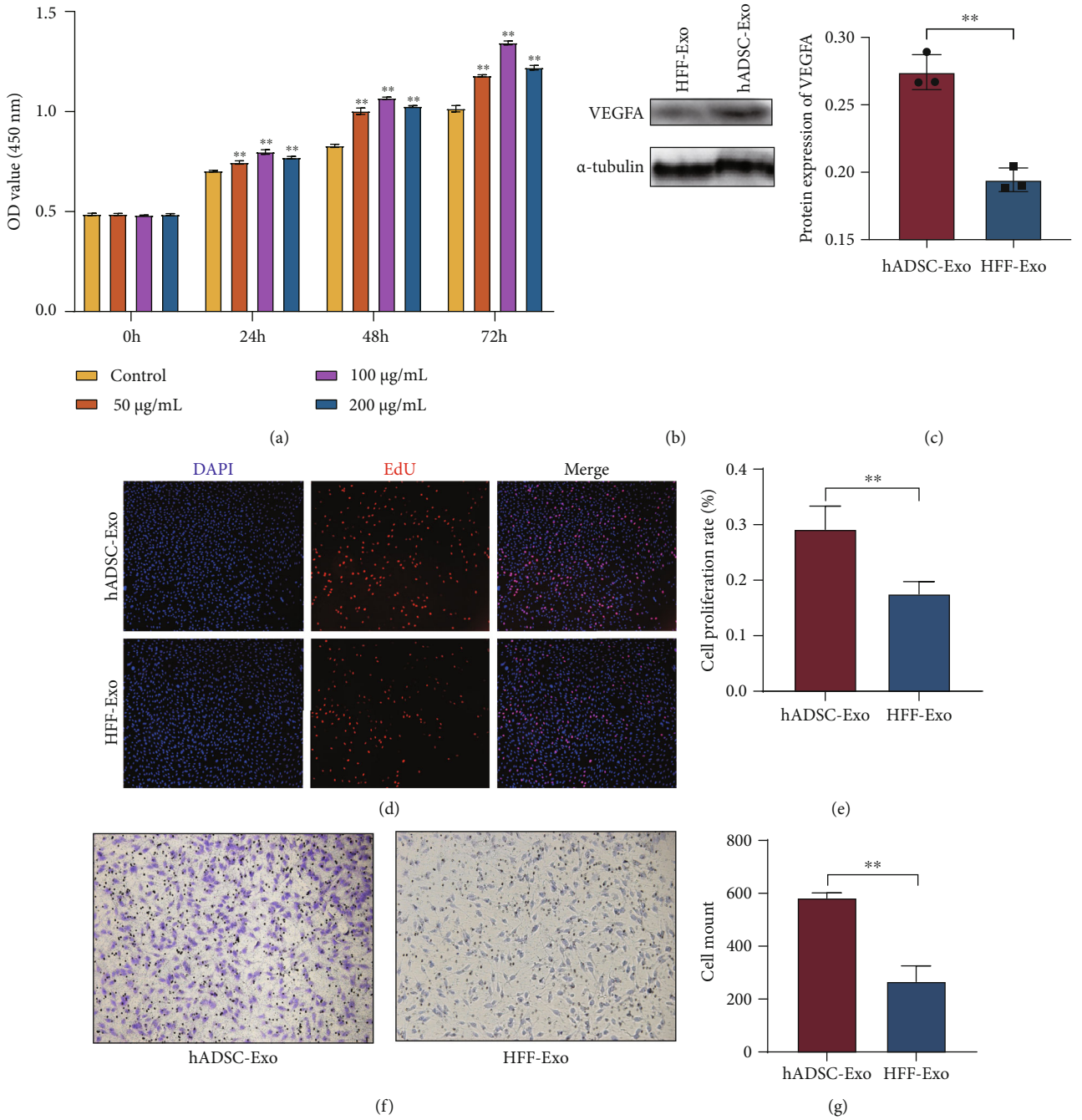

Figure 4: Proangiogenic effects of hADSC-Exo in vitro. (a) The CCK8 revealed that 100 μg/mL of hADSC-Exo had the most significant promotion effect on the proliferation of HUVEC, and this promotion effect persisted with increasing culture time. (b, c) The expression levels of VEGFA genes in fat grafts were treated with hADSC-Exo or HFF-Exo. (d) Proliferating HUVECs were stained with red fluorescence, and all nuclei were stained with blue fluorescence. (e) The cell proliferation rate of hADSC-Exo group was higher than that of HFF-Exo group. (f, g) The migration of HUVEC in the hADSC-Exo group was better than that in the HFF-Exo group. ($**p < 0.01$).

staining of PPARγ in fat grafts to observe the status of adipogenic differentiation. The results showed that hADSC-Exo group had evidently significant PPARγ expression compared to the HFF-Exo group ($p < 0.05$) (Figures 5(a) and 5(b)).

To explore the potential mechanisms of lipogenic differentiation of ADSC, the GSE61302 expression profiling data were downloaded from the GEO database, and 1970 DEGs were identified by differential analysis between undifferentiated human primary ADSCs and mature adipocytes (Figures 5(c) and 5(d)). And the top ten upregulated and downregulated DEGs were selected and listed in Tables 1 and 2, respectively. Combined with our previous miRNA sequencing data on hADSC-Exo and HFF-Exo, a total of 605 DEGs may be targeted by hADSC-Exo in the process of adipogenic differentiation of ADSC (Figure 5(d)).

3.5. Comprehensive Functional Analysis of the Key Genes Targeted by hADSC-Exo. PPI network is significant for

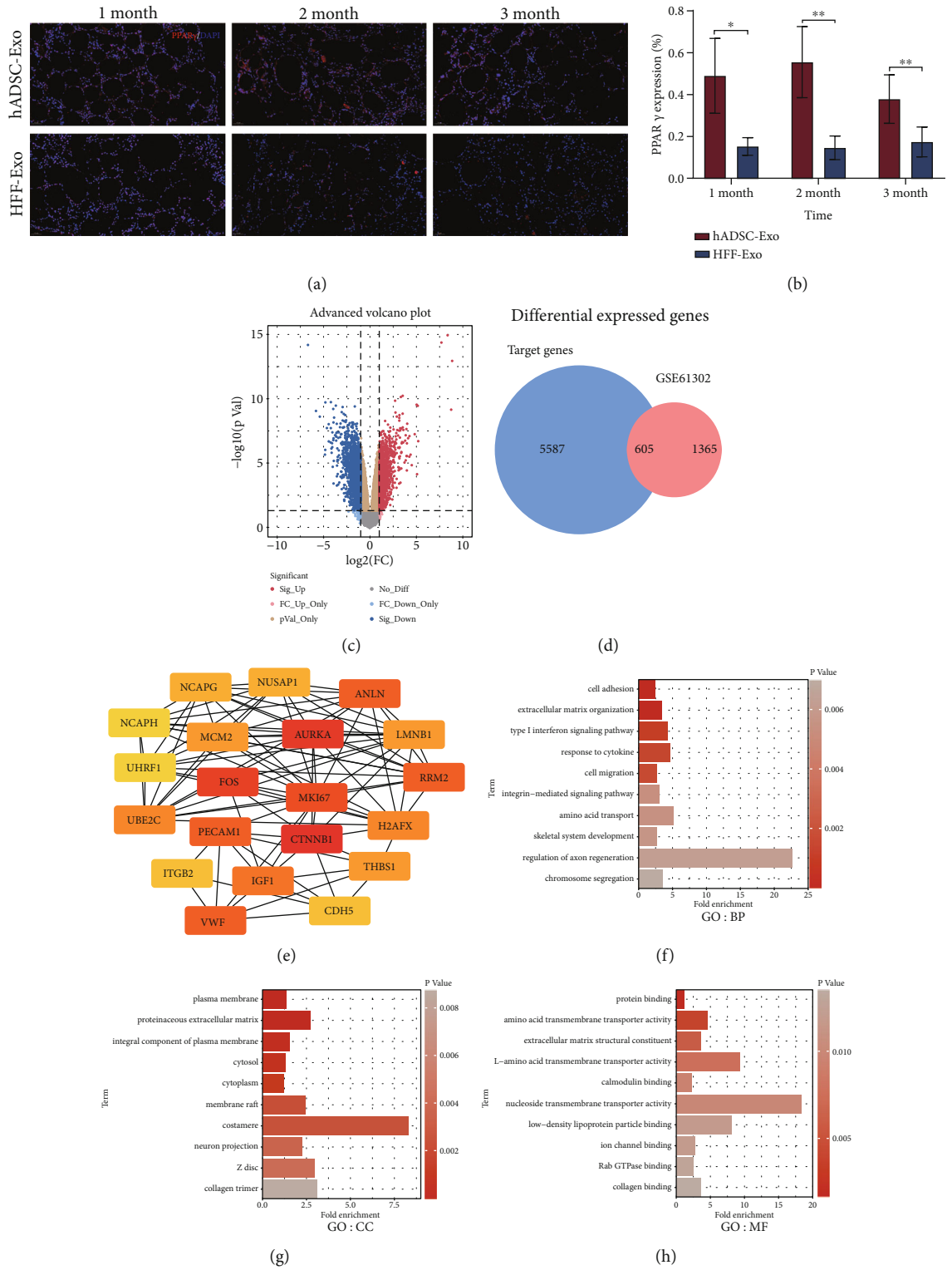

Figure 5: Comprehensive functional analysis of the key DEGs for lipogenesis targeted by hADSC-Exo. (a) The immunofluorescence staining revealed that hADSC-Exo group had evidently significant PPARγ expression compared to the HFF-Exo group. (b) The positive percentage of PPARγ expression in the fat grafts at 1, 2, and 3 months were calculated. (c) Volcano plot of the GSE61302 microarray dataset. (d) Venn diagram of key genes which may target by hADSC-Exo in the process of adipogenic differentiation of ADSC. (e) The mapped network for the top 20 hub genes in protein–protein interaction network. (f) The category of "biological process" of the target DEGs. (g) The category of "cellular component" of the target DEGs. (h) The category of "molecular function" of the target DEGs ($*p < 0.05$ and $**p < 0.01$).

Table 1: Top ten upregulated DEGs in GSE61302 between undifferentiated human primary ADSCs and mature adipocytes.

ID	Gene name	Adj. p value	p value	t	B	logFC
224354_at	No name	1.45E-09	1.15E-13	3.37E+01	1.91E+01	8.85E+00
234432_at	No name	2.23E-06	7.08E-10	1.65E+01	1.29E+01	8.75E+00
1555623_at	No name	5.86E-11	1.16E-15	4.88E+01	2.11E+01	8.36E+00
1561775_at	No name	1.08E-10	4.30E-15	4.40E+01	2.06E+01	7.69E+00
242625_at	RSAD2	5.69E-05	1.99E-07	1.02E+01	7.65E+00	5.20E+00
205660_at	OASL	1.49E-06	3.54E-10	1.75E+01	1.35E+01	5.15E+00
206336_at	CXCL6	1.79E-03	7.37E-05	5.78E+00	1.74E+00	5.07E+00
210797_s_at	OASL	1.46E-06	2.90E-10	1.78E+01	1.36E+01	5.02E+00
213797_at	RSAD2	2.91E-05	5.71E-08	1.14E+01	8.85E+00	4.82E+00
204533_at	CXCL10	6.79E-04	1.46E-05	6.83E+00	3.37E+00	4.50E+00

Table 2: Top ten downregulated DEGs in GSE61302 between undifferentiated human primary ADSCs and mature adipocytes.

ID	Gene name	Adj. p value	p value	t	B	logFC
202768_at	FOSB	1.11E-10	6.59E-15	-4.25E+01	2.05E+01	-6.68E+00
203394_s_at	HES1	2.64E-06	8.90E-10	-1.62E+01	1.27E+01	-5.82E+00
206115_at	EGR3	5.00E-06	2.48E-09	-1.49E+01	1.18E+01	-5.41E+00
218839_at	HEY1	2.15E-04	2.22E-06	-8.20E+00	5.27E+00	-4.85E+00
44783_s_at	HEY1	1.34E-04	9.50E-07	-8.86E+00	6.11E+00	-4.82E+00
201693_s_at	EGR1	1.08E-06	1.93E-10	-1.84E+01	1.40E+01	-4.81E+00
209189_at	FOS	1.56E-05	1.91E-08	-1.25E+01	9.89E+00	-4.64E+00
201890_at	RRM2	1.09E-05	9.49E-09	-1.33E+01	1.05E+01	-4.45E+00
216248_s_at	NR4A2	5.83E-05	2.08E-07	-1.02E+01	7.60E+00	-4.44E+00
233180_at	No name	6.39E-05	2.50E-07	-1.00E+01	7.42E+00	-4.24E+00

understanding the mechanisms of biosignal and the functional connections between proteins under specific physiological conditions. In this study, the PPI network analysis was performed on the 605 key genes through STRING database. Subsequently, the hub genes with the highest degree of the first 20 nodes were obtained by the degree algorithm (Table 3) and visualized with Cytoscape software (Figure 5(e)). Regarding these hub genes, CTNNB1, AURKA, FOS, MKI67, and VWF were the top five hub genes.

Furthermore, GO annotation and KEGG pathway analysis were performed to further reveal the enrichment status of the DEGs in BP, MF, CC, and KEGG pathways through the DAVID database. With regard to the "biological process" category (Figure 5(f)), it was demonstrated that most of the DEGs were mainly involved in extracellular matrix organization, cell adhesion, and type I interferon signaling pathway. Regarding the CC (Figure 5(g)), the majority of the DEGs were mainly components of plasma membrane, proteinaceous extracellular matrix, integral component of plasma membrane, and costamere. Regarding the MF (Figure 5(h)), the DEGs were mostly involved in amino acid transmembrane transporter activity, extracellular matrix structural constituent, L-amino acid transmembrane transporter activity, and protein binding. Then, the KEGG pathway analysis contributes to study the pathways and functions of the key genes (Figure 6(a)), which mainly were significantly enriched in

Table 3: Top 20 hub genes in the PPI networks.

Rank	Name	Score
1	CTNNB1	80
2	AURKA	37
3	FOS	36
4	MKI67	33
5	VWF	32
5	PECAM1	32
5	RRM2	32
5	ANLN	32
9	IGF1	30
10	H2AFX	29
10	UBE2C	29
12	THBS1	28
12	LMNB1	28
12	MCM2	28
15	NCAPG	27
15	NUSAP1	27
17	ITGB2	26
17	CDH5	26
19	NCAPH	25
19	UHRF1	25

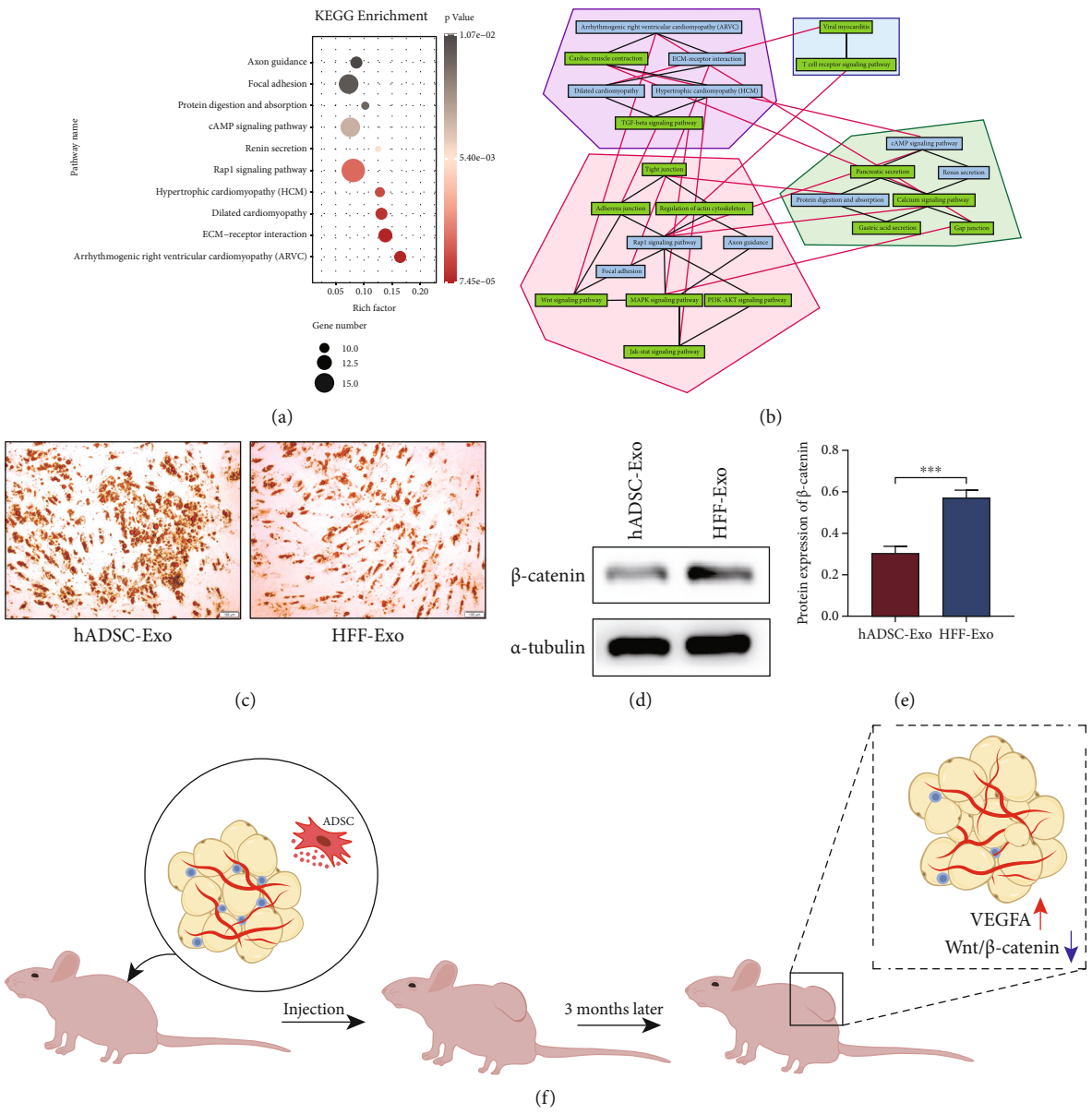

Figure 6: hADSC-Exo promoted adipogenic differentiation of ADSC in adipose grafts. (a) The category of "KEGG enrichment" of the target DEGs. (B) Clusters of pathways each one presented by a different color. The black lines are the connections between nodes that form the identified lusters, while the red ones are those excluded by the algorithm. (c) Cell adipogenic differentiation assays showed that hADSC-Exo significantly promote ADSC adipogenic differentiation. (d, e) The expression levels of β-catenin in fat grafts cotransplanted with hADSC-Exo or HFF-Exo. (f) A schematic diagram shows how hADSC-Exo promotes the angiogenesis of fat grafts and maintains the adipogenic differentiation ability of ADSC to promote the retention of fat grafts.

the signal pathways of ECM-receptor interaction, focal adhesion, and Rap1 signaling pathway.

To further gain our understanding of the pathway interactions under the study, we revealed the direct connections between pathways of interesting and highlight communities (clusters) of pathways through a bioinformatics approach based on graph theory and the edge betweenness clustering algorithm [22–24]. Wnt signaling pathway was identified according to KEGG repository to be highly connected to pathways related to cell–cell adhesion (adherens junction), cell–ECM adhesion (focal adhesion), and the prosurvival pathways of Rap1, MAPK, and PI3K-AKT. (Figure 6(b)).

3.6. hADSC-Exo Promote Adipogenic Differentiation of ADSC in Adipose Grafts. Cell differentiation assays were used to further validate the specific effects of hADSC-Exo contributing to adipogenic differentiation and showed that the adipogenic differentiation capacity of ADSCs were preserved and promoted better in the hADSCs-Exo group compared with the HFF-Exo group (Figure 6(c)). The result demonstrated that hADSCs-Exo could maintain and promoted the adipogenic differentiation capacity of ADSCs.

Wnt/β-catenin pathway, a key regulatory pathway for adipogenic differentiation, was also detected in adipose grafts. Expression levels of β-catenin were significantly lower

in the hADSCs-Exo compared with the HFF-Exo group ($p < 0.05$) (Figures 6(d) and 6(e)). The result suggested that hADSC-Exo may regulate the Wnt/β-catenin pathway to improve the adipogenesis in fat grafts.

4. Discussion

Adipose tissue is particularly sensitive to the ischemic environment, where it is susceptible to necrosis and apoptosis [25]. After fat transplantation, the graft must rebuild its blood supply to survive. Unfortunately, most fat grafts are necrotic, liquefied, and resorbed due to difficulties in reestablishing blood supply [26]. Therefore, early vascular regeneration of fat grafts is promoted as an essential strategy to improve the survival of fat grafts.

Stem cell therapy has high application value in promoting vascularization, but the risk of promoting tumor formation and the difficulty of transformation technology greatly limit its clinical application [27, 28]. Therefore, the search for key active components that can be used directly by replacement stem cells and have angiogenic effects can minimize the above potential problems. Accumulating evidences show that exosomes derived from stem cells have great potential for the treatment of tissue injury, infections, and ischemic diseases [29–31]. Meanwhile, the application of stem cell exosome is free of side effects such as malignancy, risk of vascular occlusion, and immunogenicity, indicating that stem cell exosome-based regenerative therapies are safe and have promising applications [32]. Previous studies have shown that hADSC-Exo repair ischemic tissue disease through provascular regeneration, and our previous work also found that hADSC-Exo can significantly promote the angiogenesis of artificial prefabricated dermis flap [14, 33]. However, whether hADSC-Exo can improve adipose graft survival by promoting adipose graft angiogenesis and its underlying mechanism is not yet clear.

To clarify the effect of hADSC-Exo on adipose graft retention, hADSC-Exo and HFF-Exo were cotransplanted with adipose grafts under the skin of nude mice in this study and observed that hADSC-Exo have a better retention rate than HFF-Exo (Figure 2). Interestingly, we observed that the weight and volume of fat grafts decreased gradually over time in both groups, which could be attributed to the progression of fat grafting, mainly involving cysts, calcification, nodules, fat necrosis, fibrosis, and survival, and eventually stabilization [26, 34, 35]. In addition, fat number and integrity of adipose grafts were significantly higher in the hADSC-Exo group than in the HFF-Exo group (Figures 3(a) and 3(b)). Then, CD31+ and Ki67 immunohistochemistry were used to observe the degree of graft vascularization and the number of proliferating cells and showed that hADSC-Exo group had better vascularization and proliferating cell numbers compared to the HFF-Exo group. Notably, hADSC-Exo was also able to promote the proliferation and migration of HUVEC in vitro, and the protein expression extent of VEGFA was also more significant in adipose grafts in the hADSC-Exo group, which indicates that hADSC-Exo could promote the neovascularization of fat grafts, suggesting that hADSC-Exo could improve retention by promoting graft vascularization.

Adipose tissue is rich in ADSC, which is more resistant to ischemia and hypoxia at the early stage of fat grafting compared with mature adipocytes, vascular endothelial cells, and other and blood-derived cells [36], and is able to regenerate adipose tissue through adipogenesis, angiogenesis, and paracrine effects [37]. In the study, we observed better adipose retention and adipose integrity in the hADSC-Exo group, the ability of hADSC-Exo to promote adipogenic differentiation of ADSC and thus replenish adipose grafts that aroused our interest. Peroxisome proliferator–activated receptor gamma (PPARγ), a major regulator of adipogenesis, plays a dominant role in adipocyte differentiation and acts as a transcription factor expressed in mature adipocytes [38]. Numerous studies have shown that adipose cells apparently cannot form without PPARγ [39–41]. Therefore, we observed the immunofluorescence staining result of PPARγ in two groups of fat grafts and indicated that hADSC-Exo can significantly promote the formation of adipocytes in fat grafts. Indeed, activation of adipose stem cells promotes the survival of fat cells in the regenerated area [26]. However, the potential mechanisms by which hADSC-Exo promote adipogenic differentiation of ADSC are not yet clear.

We hypothesize that hADSC-Exo may target key genes regulating pro-ADSC lipogenic differentiation through its enriched miRNAs. To clarify the potential mechanism, the gene expression microarray datasets of hADSC during in vitro adipogenic differentiation (GSE61302) were downloaded from the GEO database in our study, and 1970 DEGs were identified by differential analysis between undifferentiated human primary ADSCs and mature adipocytes. Meanwhile, our previous study found a total of 43 DE-miRNAs between hADSC-Exo and HFF-Exo. Subsequently, we made an intersection between the target genes predicted by DE-miRNAs and the DEGs to basically identify the key DEGs that may be affected by hADSC-Exo in the process of adipogenic differentiation of ADSC. Then, we explored the biological functions of these genes in adipogenic differentiation through GO annotation and KEGG pathway enrichment analysis. Interestingly, these key genes were directly related to the biological processes of extracellular matrix organization and mainly involved in the signal pathways of ECM-receptor interaction, focal adhesion, and Rap1 signaling pathway. ECM is a multifunctional network, consisting of all kinds of proteins, proteoglycans, and glycosaminoglycans, that regulates stem cell differentiation through reciprocal interactions between cells and components of the ECM [42–44]. The development of preadipocytes to mature adipocytes is accompanied by the remodeling and specific changes of ECM in the process of adipogenic differentiation [45]. Yeung et al. [46] found that the inactivation of Rap1 signaling pathway can promote adipocyte differentiation.

Hub genes as high node degree genes in the PPI network may also play an important role in the development of adipogenic differentiation. Through the analysis of the node degree algorithm in the PPI network, several hub genes with high degree were obtained. Notably, CTNNB1, the largest degree gene in the network, encodes beta-catenin 1 protein

and is also a key regulator of Wnt signaling that interacts with E-cadherin and actin cytoskeleton to mediate cell–cell adhesion [47]. Previous studies showed that the activation of Wnt/β-catenin signaling can suppress the adipogenic differentiation [48, 49]. In addition, Reggio et al. observed that WNT5a repressed PPARγ expression and adipogenesis through the activation of β-catenin signaling [50]. Numerous studies have shown that the activation of PPARγ is associated with a reduction in β-catenin levels [51, 52]. Consistently, our study found downregulation of β-catenin expression and upregulation of PPARγ in hADSC-Exo cotransplanted adipose grafts. Finally, a graph clustering approach was used to expand the relationship between pathways and confirmed our line of investigation demonstrating that Wnt signaling pathway are highly connected to the pathways involved in cell–cell adhesion (adherens junctions), cell–ECM adhesion (focal adhesion), and are also being connected to the prosurvival pathways of Rap1, MAPK, and PI3K-AKT. Our results provide new insights into the molecular mechanism underlying the role of ADSC-Exo in the survival of fat grafts (Figure 6(f)). However, there were also certain limits in our study. For example, there did not involve the specific miRNA engaged in this pathway in hADSC-Exo; thus, the targeting mechanism is not enough strong. Whereafter, we will use single-cell sequencing technology to compare the cellular and molecular components involved in regeneration in fat grafts, so as to further explore the mechanism of fat graft survival.

5. Conclusion

In summary, our findings suggest that hADSC-Exo promotes the angiogenesis of fat grafts and maintains the adipogenic differentiation ability of ADSC to promote the retention of fat grafts and provides a theoretical basis for the clinical translation of hADSC-Exo in fat grafting.

Data Availability

The next-generation sequencing data used to support the findings of this study were supplied by Yuchong Wang under license and so cannot be made freely available.

Conflicts of Interest

The authors have declared that no competing interest exists.

Authors' Contributions

Kexin Chen, Jiachao Xiong, and Sha Xu contributed equally to this work. Kexin Chen and Jiachao Xiong designed, performed experiments, analyzed data, and wrote the manuscript. Sha Xu and Minliang Wu performed experiments. Chunyu Xue and Chuan Lv edited manuscript. Yuchong Wang and Minjuan Wu conceived, designed experiments, and reviewed/edited the manuscript. And all authors approved the final version of the manuscript.

Acknowledgments

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Review Article

Insight in Hypoxia-Mimetic Agents as Potential Tools for Mesenchymal Stem Cell Priming in Regenerative Medicine

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Hypoxia-mimetic agents are new potential tools in MSC priming instead of hypoxia incubators or chambers. Several pharmaceutical/chemical hypoxia-mimetic agents can be used to induce hypoxia in the tissues: deferoxamine (DFO), dimethyloxaloylglycine (DMOG), 2,4-dinitrophenol (DNP), cobalt chloride ($CoCl_2$), and isoflurane (ISO). Hypoxia-mimetic agents can increase cell proliferation, preserve or enhance differentiation potential, increase migration potential, and induce neovascularization in a concentration- and stem cell source-dependent manner. Moreover, hypoxia-mimetic agents may increase HIF-1α, changing the metabolism and enhancing glycolysis like hypoxia. So, there is clear evidence that treatment with hypoxia-mimetic agents is beneficial in regenerative medicine, preserving stem cell capacities. These agents are not studied so wildly as hypoxia but, considering the low cost and ease of use, are believed to find application as pretreatment of many diseases such as ischemic heart disease and myocardial fibrosis and promote cardiac and cartilage regeneration. The knowledge of MSC priming is critical in evaluating safety procedures and use in clinics. In this review, similarities and differences between hypoxia and hypoxia-mimetic agents in terms of their therapeutic efficiency are considered in detail. The advantages, challenges, and future perspectives in MSC priming with hypoxia mimetic agents are also discussed.

1. Introduction

The proper functioning of human tissues and organs depends on natural regeneration processes. The regenerative potential is primarily maintained by the stem and progenitor cells whose progeny replace aged or injured cells when needed [1–3]. Mesenchymal stem cells (MSCs) are stromal cells that self-renew and display multipotency, together with unique immunomodulatory properties. Numerous studies are currently carried out on MSCs to treat neurodegenerative or immune-derived inflammatory diseases [1, 4, 5]. MSCs can be isolated from adult tissues (e.g., bone marrow (BM), adipose tissue (AD), skeletal muscle (SM), and dental pulp (DP)) [6–9] or fetal tissues (e.g., placenta, amniotic fluid (AF), Wharton jelly (WJ), and umbilical cord (UC)) [10, 11]. Epidermal stem cells, multipotent skin-derived precursors, and other stem cells can also be efficiently isolated from human skin [12].

By July 2020, 1,138 clinical trials have been registered at clinicaltrials.gov [13], mostly in traumatology, pneumology, neurology, cardiology, and immunology [14–19]. Most registered cases were in phases 1 (Ph1) and 2 (Ph2) of clinical trials. The percentage of particular phases of clinical trials in the fields mentioned above is as follows: in traumatology (total 234 cases), 30.7% in Ph1, 58.5% in Ph2, 9.8% in Ph3, and 0.8% in Ph4; in pneumatology (total 99 cases), 43.4% in Ph1, 53.5% in Ph2, 3.0% in Ph3, and 0% in Ph4; in neurology (total 97 cases), 31.9% in Ph1, 62.8% in Ph2, 4.1% in Ph3, and 1.0% in Ph4; in cardiology (total 83 cases), 25.5% in Ph1, 60.2% in Ph2, 14.4% in Ph3, and 0% in Ph4; and in immunology (total 78 cases), 17.9% in Ph1, 64.1% in Ph2, 17.9% in Ph3, and 0% in Ph4 [13, 16]. The outcomes

of 18 clinical tests have already been described [16], and bone marrow was the most common source of isolated cells. MSCs have found potential applications in the treatment of multiple sclerosis (MS), Crohn's disease (CD), diabetes mellitus (DM), graft-versus-host disease (GVHD), rejection after liver transplant, liver disorders [5], and acute and chronic wounds [20, 21].

Despite such notable progress, there are still numerous challenges. Clinical applications demand systemic administration of the high number of stem cells (50–200 million per patient) [16, 22]. The number of stem cells in human tissues is usually small [23], and their efficient proliferation *in vitro* is challenging [24–26]. Both MSC aging and spontaneous differentiation are factors that may occur *in vitro*. The isolated stem cells are usually grown *in vitro* under ambient conditions where oxygen concentration is four to ten times higher than in a stem cell niche [27–30]. Thus, high oxygen concentration upon MSC culture results in early senescence and nuclear damage and may increase the doubling time [31–33]. Poor MSC engraftment after transplantation was also revealed [34].

Over the last few years, numerous low oxygen priming approaches have been explored for MSC clinical application [35, 36]. MSCs growing under hypoxia [37] demonstrate enhanced proliferation, immunomodulatory properties [38–43], efficient survival, and neovascularization after grafting.

As such, several hypoxia-mimetic agents can be used to induce hypoxia in tissues, e.g., deferoxamine (DFO), dimethyloxaloylglycine (DMOG), 2,4-dinitrophenol (DNP), cobalt chloride ($CoCl_2$), and isoflurane (ISO). Could they effectively replace the hypoxia chambers/incubators in the MSC priming? This review looks for an answer to this question and discusses similarities and differences between the effects of hypoxia and hypoxia-mimetic agents. The oxygen concentration, incubation time, and MSC therapeutic proficiency are described in detail. Since there are still some ambiguities in the literature regarding hypoxia as a standard approach in MSC production, this issue will be extensively discussed.

To summarize the recent findings on hypoxia in this review, we searched PubMed, Scopus, Science Direct, and Web of Science databases from 2006 to September 2021 for potentially relevant studies published in English. Original papers, systematic reviews, and book chapters were reviewed. The search strategy first has focused on critical terms: hypoxia, hypoxia mimetic agents, mesenchymal stem cells, and clinical applications of MSC. These criteria have been extended with the more detailed terms: application in regenerative medicine, cell treatment, cell-based therapies, mesenchymal cells' source (Warton jelly, umbilical cord, bone marrow, umbilical cord blood, adipose, and dental pulp originated from human, rat, and mouse), and chemicals: deferoxamine, cobalt chloride, isoflurane, dimethyloxaloylglycine, and 2,4-dinitrophenol. We excluded studies enrolling hypoxia/hypoxia-mimetic agents together with specific adjuvants such as immunomodulators.

2. Role of Hypoxia in a Stem Cell Niche

The stem cell niche is a microenvironment, which governs stem cell's functions and fate [44]. Morphogens, growth factors, cytokines, oxygen tension, extracellular matrix, and shear stress could affect stem cells within the niche [5, 45].

MSCs can be found in the niches close to blood capillaries throughout the body [46]. The oxygen concentration in the tissues where MSCs reside is low despite their efficient vascularization [47, 48]. The oxygen concentration is much lower in human tissues than in inhaled air (21%). It happens because the oxygen concentration of the inhaled air constantly drops, entering the lungs, and when it reaches organs and tissues, its concentration ranges from 2% to 9% [49, 50]. Since the concentration of O_2 in blastocysts and stem cells niches is very low, oxygen tensions tend to be critical in their metabolic milieu. Hypoxia sustains the phenotype of hematopoietic, embryonic, neural, and mesenchymal stem cells and influences stem cells' function and fate. Furthermore, hypoxia acts on stem cells via different molecular pathways, including signaling of homolog translocation-associated (Drosophila) (Notch) and octamer-binding transcription factor 4 (Oct4), the stemness controllers [25].

Stem cells are physiologically adapted to hypoxia. Therefore, hypoxic priming should maintain MSCs in an undifferentiated state and preserve their functions and plasticity.

3. Hypoxia versus Hypoxia-Mimetic Agents for MSC Priming

Injured tissues have poor vascularization (especially in ischemic injuries) and cannot maintain the metabolism of implanted not-primed MSC at an appropriate rate; therefore, most cells undergo apoptosis soon after transplantation. It is due to stem cells grown in normoxia not adapting quickly to the conditions of hypoxia. Hence, to survive after transplantation, stem cells must be trained ex vivo to sustain hypoxia conditions [51].

The simplest solution is to cultivate MSCs under low oxygen conditions. Various hypoxia incubators and chambers were used for MSC culture. However, both have limitations in their use [52]. They suggest that pharmaceutical/chemical agents are more valuable because they provide higher oxygen tension stability than hypoxic chambers and are not expensive [53].

Now the question arises whether pharmacological or chemical hypoxia-mimetic agents act similarly on stem cells. Before answering this question, we intend to discuss the influence of hypoxia on the crucial MSC features.

3.1. Cell Surface Markers and Morphology. The most important MSC feature is their immunophenotype that defines their stemness according to the International Society for Cellular Therapy (ISCT). MSCs express CD90, CD105, and CD73 antigens and do not express CD11b, CD14, CD19, CD45, CD34, and CD79a antigens, nor human leukocyte antigen-DR isotype (HLA-DR). The proteins SRY-box transcription factor 2 (SOX2) and Oct4 occur in embryonic stem cell- (ESC-) like [54]. The expression of other surface markers depends on the MSC tissue source. Homeobox transcription factor NANOG, reduced expression-1 (REX-1), T cell receptor alpha locus 1-60 (TRA-1-60), TRA-1-81, stage-specific mouse embryonic antigen (SSEA-3), and

SSEA-4 markers have been found on MSCs isolated from human liver and fetal blood but not on the cells derived from adult bone marrow [55, 56].

The influence of the hypoxia priming on MSC's surface markers is summarized in Table 1. Hypoxic conditions in the oxygen range of 2-5% preserve the expression of surface markers on MSCs. Only in low oxygen concentration of 1% are the results inconclusive. The expression of negative surface markers is maintained at 1% O_2 [57–59], but some studies showed a reduced expression of positive markers. Compared to normoxia, CD44 and CD105 reduction on the MSC surface from 90% to 75% and from 99.4% to 94.9%, respectively, was noted [57]. Upregulation of other stem cell markers as Oct4, REX-2, or NANOG was presented [38, 60].

Of no less importance is maintaining the appropriate morphology of MSCs growing in confluence. While increased cellular density and number of passages significantly change MSC's morphology under normoxia and cause cell retraction at high density, hypoxic conditions retain the MSC's spindle shape, and cells can divide even at high density, permitting multilayer formation [38]. Similarly, MSCs treated with a hypoxia-mimicking agent DFO did not alter their morphology. However, some intracellular vacuole-like structures may occur within the cells [61].

To summarize, the expression of stem cell surface markers is generally preserved under hypoxia but depends on the oxygen concentration, exposure time, tissue, and donor of MSCs. Up to date, there are no data on the influence of hypoxia-mimicking agents on MSC surface markers expressions.

3.2. Viability, Proliferation, and Clonogenicity. A high proliferation rate is critical for the successful implementation of stem cell-based therapy. The oxygen concentration and the incubation time may influence the overall hypoxia effect on stem cells, especially their viability, proliferation, and clonogenicity.

3.2.1. Hypoxia. As shown in Table 2, the proliferation, viability, and clonogenicity of the stem cells derived from various tissues were studied under at least 30 conditions different in terms of oxygen concentration and incubation time under reduced oxygen concentration.

In 19 conditions, an increase in proliferation or clonogenicity of MSCs was observed in the oxygen concentration ranged from 1 to 5%. Out of these 30 conditions considered, a decrease in cell viability was recorded in eight. This discrepancy is not related to oxygen concentrations since proliferation inhibition was observed at both 1% and 5% oxygen concentrations. It also does not depend on the time of cell growth under hypoxia because inhibition of proliferation was observed both after 2-day exposure to reduced oxygen concentration and after 21-day exposure at similar oxygen concentrations. These divergent effects can also be seen on one type of stem cell, e.g., BM-MSCs. Likewise, the impact of the test method on the results obtained cannot be attributed, e.g., Trypan Blue staining and counting cells under the microscope were used at the elaboration of conditions resulting in discrepant observations. It is, therefore, possible that more subtle molecular phenomena occurring in stem cells while growing under hypoxic conditions should be investigated, such as transcriptome or metabolome of hypoxia-treated cells.

The higher proliferation of MSCs could be attributed to the transition from aerobic to anaerobic respiration through oxidative phosphorylation and glycolysis, respectively [67]. The increase in glucose consumption and lactate generation in UC-MSCs in hypoxic culture may exemplify the metabolic changes described above and require enhanced glucose transport into the cells. Increased MSC proliferation under hypoxia enhances glucose uptake as the critical carbon source for the biosynthesis of essential nutrients. The involvement of metabolic pathways as glycolysis (lactate dehydrogenase A, LDHA), oxidative phosphorylation (3-phosphoinositide-dependent protein kinase 1, PDK-1), cellular glucose transport (cellular glucose transporter-1, GLUT-1), pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G6PD), and the significant targets of hypoxia-inducible factor 1 alpha (HIF-1α) transcriptional factors was demonstrated in the diminished oxygen concentration [63, 68, 69].

HIF-1α is a crucial transcriptional factor, which regulates the adaptive response to hypoxia. Many proteins react directly with HIF-1α enhancing or reducing their activities. HIF-1α stabilization improves MSC's proliferation rate and may augment their therapeutic potential [70].

The reduction of cellular senescence and inhibition of the telomere shortening was also observed in MSCs under hypoxic conditions [62, 63, 71, 72]. The mechanism of apoptosis suppression under hypoxia might relate to the cellular tumor antigen (p53) pathway inhibition [73]. The decreased O_2 tension could also lead to the lower level of reactive oxygen species (ROS); the primary factor was attributed to increased cellular damage [74].

(1) MSC's Gene Expression. Cells' adaptation to hypoxia requires changes in molecular pathways. Hypoxia regulates the transcription of hundreds of genes, which play a role in oxygen-dependent functions like angiogenesis, glycolysis, metabolism, proliferation, and apoptosis [75]. Most of these changes are HIF-1α-dependent and transcriptionally regulated. HIF-1α is also subject to epigenetic mechanisms such as histone modification, DNA methylation, and noncoding RNA-associated gene silencing [76]. Thus, epigenetic modifications are additional mechanisms regulating gene expression in hypoxia and enhancing or inhibiting their activity. However, the contribution of microRNA (miRNA) functioning during hypoxia and DNA methylation is not yet fully understood [77]. Furthermore, molecules of short noncoding RNAs and miRNAs, which regulate gene expression, are controlled by hypoxia in stem cell niches [78]. Some miRNA regulate vascular endothelial growth factor (VEGF), which stimulates angiogenesis and tightly controls hypoxia-induced cellular alteration [77, 79].

Beyond epigenetic mechanisms, hypoxia upregulates over 135 genes governing several physiological pathways, e.g., glycolysis, metabolism, proliferation/survival,

Table 1: Effect of hypoxia on MSC surface markers.

Treatment conditions		Types of stem cells	The effect compared to normoxia (method of analysis)	Ref.
O_2 concentration	Time/passage			
1%	2 d	hBM-MSC	No changes in positive (CD44 and TF) and negative markers (CD11b, CD19, CD34, CD45, and HLA-DR); decreased level of CD73, CD90, and CD105 (flow cytometry)	[57]
1%	10 d	hBM-MSC	No changes in positive (CD73, CD90, and TF) and negative markers (CD45, CD34, Cd11b, CD19, and HLA-DR); decreased level of CD105 and CD44 (flow cytometry)	[57]
1%	14 d	hBM-MSC	No changes in positive (CD73, CD90, CD105, CD106, CD 146, and MHC class I) and negative markers (CD45, CD34, and HLA-DR) (flow cytometry)	[58]
1%	21 d	hAD-MSC	No changes in positive (CD90 and CD105); increased level of negative markers (CD34, CD54, and CD 166) (flow cytometry)	[62]
2%	2 d	hBM-MSC	No changes in positive (CD73, CD44, CD90, and CD105) and negative markers (CD11b, CD19, CD45, CD34, and HLA-DR) (flow cytometry)	[57]
2%	12 d	hBM-MSC	No changes in positive (CD90, CD73, and CD105) and negative markers (CD31, CD34, CD45, and CD80) (flow cytometry)	[63]
2%	7 passages	hBM-MSC	Upregulation of *Oct4* and *REX-1* (RT-PCR)	[38]
2%	10 passages	hWJ-MSC	No changes in positive (CD73, CD105, CD90, CD44, CD10, CD29, and CD13) and negative markers (CD14, CD34, CD33, CD45, and HLA-DR) (flow cytometry); increased level of *DNMT3B*, *CRABP2*, *IL6ST*, *IFITM1*, *GRB7*, *IMP2*, *LIN28*, and *KIT* (RT-PCR)	[64]
2.5%*	>72 h*	hUCB-MSC	No changes in positive (CD44, CD73, CD90, and CD105) and negative markers (CD14, CD34, CD45, CD271, and HLA-DR) (flow cytometry); upregulation of *Oct4*, *NANOG* (RT-PCR)	[60]
2.5%*	>72 h*	hUCB-MSC	No changes in positive (CD73, CD44, CD105, and CD90) and negative markers (CD14, CD45, and CD106) (flow cytometry)	[65]
5%	2 d	hBM-MSC	No changes in positive (CD44, CD90, and CD73) and negative markers (CD11b, CD19, CD34, CD45, and HLA-DR); reduced level of CD105 (flow cytometry)	[57]
5%	14 d	hBM-MSC	No changes in positive (CD73, CD90, CD44, CD105, and STRO-1) and negative markers (CD34); reduced level of CD146 and CD45 (flow cytometry)	[65]
5%	Primary cells and passage 2	hBM-MSC	No changes in positive (CD29, CD73, CD90, CD44, CD105, and STRO-1) and negative markers (CD45 and CD34) (flow cytometry)	[66]

*Hypoxic preconditioning in 2.5% O_2 for 15 minutes, then reoxygenation at 21% O_2 for 30 minutes, and again hypoxia preconditioning at 2.5% O_2 for 3 days; h: human; d: day/days; UCB: umbilical cord blood.

transduction, and signaling transduction in BM/umbilical cord blood- (UCB-) MSCs in the oxygen range from 1.3% to 10% [75, 82]. Short-term hypoxia downregulates proapoptotic genes such as BCL-2-associated X (*BAX*), B-cell lymphoma 2 (*BCL-2*), and caspase 3 (*CASP-3*) (Table 2), thus preventing cells from cellular damage after transplantation [57].

Hypoxic conditions (1-5% O_2) increased expression of *HIF-1α* in BM-, UCB-, umbilical cord (UC), and WJ-MSCs [58, 64, 69]. Only Antebi et al. noted a downregulation of *HIF-1α* under 1% hypoxia in BM-MSCs [57]. Upregulation of energy metabolism-related genes *GLUT-1*, *PDK-1*, and *LDH* was noticed in 1.5, 2.5, and 5% O_2 [69]. Overexpression of *Slc16a3* (a gene of monocarboxylate transporter-4, MCT-4) under prolonged hypoxia in mBM-MSC was noted [68]. The expression of the proliferative/survival genes *Vegf-d*, placental growth factor (*Pgf*), and matrix metalloproteinase 9 (*MMP-9)* was also elevated in 1.3 and 10% O_2 compared to normoxia [75]. The rise in *Notch*, Notch ligand, and *JAGGED* was observed, suggesting a link between hypoxia and Notch signaling pathway. Moreover, the augmented proliferation of hWJ-MSCs under hypoxia confirms the Notch-related proliferation [64].

(2) Reoxygenation of MSCs in Culture. As mentioned in Table 2, there is another way to grow cells with limited oxygen availability. It includes 15 min of preconditioning at 2.5% O_2, 30 min of reoxygenation in ambient conditions, and the final conditioning at 2.5% O_2 for 72 h. Such conditions were used for hUB-MSC culture, significantly improving the cell proliferation and migration *in vitro*.

The reoxygenation process following short hypoxia priming enhanced the prosurvival genes' expression together with numerous angiogenic and trophic factors, such as the basic fibroblast growth factor (bFGF) and VEFG in MSCs [18, 60, 84, 86]. Moreover, other positive effects include the reduced release of lactate dehydrogenase, lower activity of apoptosis-related caspases, and diminished cell sensitivity to ischemia resulting from the reoxygenation of the MSC culture [57, 87].

Table 2: Effect of hypoxia on MSC viability, proliferation, and clonogenicity.

Treatment conditions		Types of stem cells	The effect compared to normoxia (methods of analysis)	Ref.
O_2 concentration	Time/ passage			
1%	2 d	hBM-MSC	Proliferation (DNA Quant-iT Picrogreen assay), clonogenicity (Giemsa staining), and metabolic activity (Vybrant assay) increased; *HIF-1α* downregulated (qRT-PCR), the proapoptotic genes: *BAX*, *BCL-2*, and *CASP-3* downregulated (qRT-PCR)	[57]
1%	2 d	rBM-MSC	The proliferation decreased (Trypan Blue staining, cell count)	[80]
1%	7 d	hBM-MSC	Proliferation significantly reduced (MTS proliferation assay)	[58]
1%	7 d	hBM-MSC	*HIF-1α* upregulated three-folds (qRT-PCR)	[58]
1%	9 d	hAD-MSC	Proliferation increased 1.7-folds (Trypan Blue staining, cell count)	[62]
1%	10 d	hBM-MSC	Proliferation (DNA Quant-iT Picrogreen assay) decreased, and metabolic activity increased (Vybrant assay), *HIF-1α* downregulated (qRT-PCR), the proapoptotic genes *BCL-2* and *CASP-3* downregulated, *BAX* upregulated (qRT-PCR)	[57]
1%	14 d	mBM-MSC	Viability (MTT viability assay) and proliferation (BrdU cell proliferation assay) increased, the main metabolic regulators like *Hk2* upregulated (sqRT-PCR), shift to anaerobic glycolysis, the *Slc16a3* (MCT-4) gene upregulated under prolonged hypoxia (qRT-PCT), the MCT-4 level increased under prolonged hypoxia (WB)	[68]
1%	14 d	rBM-MSC	Clonogenicity increased (crystal violet staining)	[80]
1%	21 d	hAD-MSC	Cell aging reduced, telomeres longer 1.5-folds (qPCR)	[62]
1%	21 d	hBM-MSC	A slowdown of cell cycle progression, accumulation in G1 phase under prolonged hypoxia (flow cytometry)	[58]
1-3%	16 h	hBM-MSC	Viability and proliferation (flow cytometry) maintained, Akt signaling pathway activated (WB)	[81]
1.5%	1 d	hBM-MSC hUCB-MSC	Proliferation increased (Trypan Blue staining, cell count) and the cell cycle faster progression (flow cytometry), HIF-1α increased (WB)	[82]
1.5%	3 d	hUC-MSC	Proliferation decreased (Trypan Blue staining, cell count), *LDHA, GLUT-1*, and *PDK-1* upregulated (RT-PCR), glutamate production decreased (HPLC), glucose consumption significantly increased (YSI 2700 analyzer)	[69]
2%	2 d	hBM-MSC	Proliferation (DNA Quant-iT Picrogreen assay), clonogenicity (Giemsa staining), and viability (flow cytometry) increased	[57]
2%	2 d	hWJ-MSC	Expression of the genes *HIF1-α*, *HIF-2α*, *Notch2*, and *JAGGED1* increased (RT-PCR)	[64]
2%	7 d	hBM-MSC	A high growth rate maintained even after confluency–multilayer formation (cell count, growth curve), *HIF-2α* upregulated (RT-PCR)	[38]
2%	7 d	hBM-MSC	Clonogenicity increased (crystal violet staining)	[63]
2%	12 d	hBM-MSC	Higher proliferation rate (Trypan Blue staining, cell count), the number of actively dividing cells significantly increased (PKH26 Red Fluorescent Cell Linker kit), the cellular division started earlier in the cell cycle (PKH26 staining, flow cytometry)	[63]
2%	20 d	hBM-MSC	Clonogenicity (colony count from microscopic images) and doubling time (cell count and growth curve) maintained, cellular senescence reduced (β-galactosidase staining, histochemistry)	[71]
2%	Passages 2-7	hBM-MSC	Higher cell number in each passage from 2 to 7 (Trypan Blue staining, cell count)	[38]
2%	10 passages	hWJ-MSC	Faster growth rates and higher total cell number yielded (cell area count, image analysis), normal karyotype maintained (Giemsa staining)	[64]
2%	64 d	hBM-MSC	Homogenous morphology of rapidly self-renewing cells maintained up to 52 d (microscopy analysis)	[83]
2.5%	3 d	hUC-MSC	Proliferation increased (cell counting under a microscope), HIF-1α increased (WB), *PDK-1, GLUT-1*, and *LDHA* upregulated (RT-PCR), glutamate production diminished (HPLC), glucose consumption significantly increased (YSI 2700 SELECT analyzer)	[69]
2.5% *	>3 d*	hUCB-MSC	Cell viability (at 24 h and 2 d) increased (Trypan Blue staining, cell count, and MTT); proliferation (at 3 d) increased (Trypan Blue staining, cell count), CFU-F number *in vitro* significantly enhanced (Giemsa staining)	[60]
2.5% *	>3 d*	hUCB-MSCs		[84]

Table 2: Continued.

Treatment conditions		Types of stem cells	The effect compared to normoxia (methods of analysis)	Ref.
O_2 concentration	Time/ passage			
			Cell metabolic activity (MTT), CFU-F number (Giemsa staining), and proliferation (at 2 and 3 d) (Trypan Blue staining, cell count) increased, doubling time reduced (at 2 and 3 d) (Trypan Blue staining, cell count), cell death inhibited (at 2 and 3 d) (microscope analysis)	
3%	~100 d Passage 1	hBM-MSC	Proliferative lifespan with additional 10 PD improved (flow cytometry), transcription of hypoxia-related the genes encoding *VHL*, *HIF-1*, *PH-4*, *HYOU1*, *HIF1AN*, *HIG*, and *HIG* unaltered (qPCR)	[33]
3%	Over 25 passages	hBM-MSC	Cell growth improved (Trypan Blue staining, cell count), population doublings increased (Trypan Blue staining, cell count), oxidative stress reactions (DHE, flow cytometry) and nuclear alterations such as damage of DNA, telomere shortening, and chromosomal abnormalities (DAPI, Q-FISH, Breast Aneusomy Multicolor Probe kit) limited, glycolysis increased (OCR/ECAR, F96 Flux analyzer)	[32]
5%	2 d	hBM-MSC	Proliferation rate lowered (DNA Quant-iT Picrogreen assay), clonogenicity (Giemsa staining), and metabolic activity elevated (Vybrant assay)	[57]
5%	3 d	hUC-MSC	Proliferation increased (Trypan Blue staining, cell count), *LDHA*, *PDK-1*, and *GLUT-1* upregulation (RT-PCR)	[69]
5%	4 d	rBM-MSC	Proliferation rate increased (flow cytometry)	[85]
5%	4 d	hBM-MSC	Clonogenicity (crystal violet staining), proliferation (EDU Proliferation kit), and metabolic activity (Alamar Blue staining) increased	[65]
5%	14 d	hBM-MSC	Clonogenicity decreased at primary cells and the passage 1 but increased at the passages 2 and 3 (crystal violet staining)	[66]
5%	20 d	hBM-MSC	Colony formation significantly reduced (colony count from microscopic images), doubling time maintained (Trypan Blue staining, cell count, growth curve), cellular senescence reduced (β-galactosidase staining, histochemistry, blue stained cell count)	[71]
5%	Passage 1-10	hBM-MSC	The number of population doublings increased (Trypan Blue staining, cell count), cellular senescence reduced (β-galactosidase staining, histochemistry, blue stained cell count)	[72]

*Hypoxic preconditioning in 2.5% O_2 for 15 minutes, then reoxygenation at 21% O_2 for 30 minutes, and again hypoxia preconditioning at 2.5% O_2 for 3 days; d: day/days; h: human; m: mouse; r: rat; PD: population doublings; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BrdU: 5-bromo tetrazolium inner salt-20-deoxyuridine; MTS: tetrazolium inner salt; WB: Western Blotting.

Table 3: Effect of pharmaceutically and chemically derived hypoxia on MSC viability, proliferation, and clonogenicity.

Treatment conditions		Stem cell type	The effect compared to normoxia (methods of analysis)	Ref.
An agent/ concentration	Time			
DFO/0.1 μM	53 d	hBM-MSC	Proliferation increased (Incucyte HD Imaging system)	[61]
DFO/10 μM	2 d	hBM-MSC	The genes related to glycolysis (*HK2*, *PDK-1*, *BNIP3*, *LDHA*), viability, and survival upregulated (microarray analysis)	[61]
DFO/10 μM	53 d	hBM-MSC	Proliferation inhibited at concentrations of 10 μM and higher (Incucyte HD Imaging system)	[61]
DFO/50 μM	12 h	hBM-MSC	Proliferation as effective as for 2 d in 3 μM DFO (Incucyte HD Imaging system), HIF-1α upregulated (microarray analysis)	[99]
DFO/50 μM	1-3 d	rBM-MSC	Viability increased (MTT)	[100]
DFO/50-500 μM	1 d	hAD-MSC	Viability unchanged (CellTiter 96 Aqueous kit)	[102]
DFO/100 μM	12 h	rBM-MSC	*HIF-1α* increased (qRT-PCR)	[100]
DFO/100 μM	1 d	rBM-MSC	HIF-1α increased (WB)	[100]
DFO/100 μM	1-3 d	rBM-MSC	Viability increased (MTT)	[100]
DFO/100 μM	2 d	hWJ-MSC	HIF-1α increased (WB)	[115]
DFO/120 μM	2 d	hUC-MSC	Cell viability was DPO concentration-dependent, cell viability decreased above 120 μM DFO (MTT)	[97]
$CoCl_2$/50-300 μM	1 d	hAD-MSC	Viability increased (MTT)	[102]
$CoCl_2$/100 μM	1-2 d	hDP-MSC hUC-MSC hAD-MSC	Viability increased (MTT)	[53]
$CoCl_2$/100 μM	2 d	hDP-MSC hUC-MSC hAD-MSC	HIF-1α increased in DP- and UC-MSC and maintained in AD-MSC (WB)	[53]
$CoCl_2$/100 μM	2 d	hUC-MSC	Viability decreased above 100 μM $CoCl_2$ (MTT)	[97]
$CoCl_2$/100 μM	6 d	Coculture hBM-	The higher proliferation of hBM-MSC in coculture (crystal violet staining), reduced viability of hBM-MSC	[52]

Table 3: Continued.

Treatment conditions		Stem cell type	The effect compared to normoxia (methods of analysis)	Ref.
An agent/ concentration	Time			
$CoCl_2$/0.5 mM	1 d	MSC HUVEC hAD-MSC	Reduced viability (MTT)	[102]
DMOG/100 μM +SD	1 d	rBM-MSC	Proliferation maintained (Trypan Blue staining, cell count), PI3K/Akt signaling activated (WB), HIF-1α increased (WB)	[103]
DMOG/0.5 mM + SD	1 d	rBM-MSC	Proliferation maintained (Trypan Blue staining, cell count), PI3K/Akt signaling activated (WB), HIF-1α increased (WB)	[103]
DMOG/0.5 mM + 1%O_2	2 d	rBM-MSC	HIF-1α increased (WB)	[80]
DMOG/0.5 mM	6 d	Coculture hBM-MSC HUVEC	The higher proliferation of hBM-MSC in coculture (crystal violet staining), increased viability of hBM-MSC	[52]
DMOG/1 mM	1 d	rBM-MSC	Viability increased in vitro (Hoechst 33342 staining), HIF-1α increased (WB), glucose transporter 1 increased (WB), the pAKT level increased (WB), increase survival of MSC after transplantation into ischemic heart (a rat model) (TUNEL assay), time-dependent protective effect against cell death in vitro (Trypan Blue staining, cell count)	[92]
DMOG/1 mM + SD	1 d	rBM-MSC	Proliferation maintained (Trypan Blue staining, cell count), PI3K/Akt signaling activated (WB), HIF-1α increased (WB)	[103]
DMOG/5 mM + SD	1 d	rBM-MSC	Proliferation decreased (Trypan Blue staining, cell count)	[103]
ISO/2%	4 h	hBM-MSC	Cell metabolic activity increased after 4 h, significantly reduced after 6 h at ISO concentrations above 2% (MTT), HIF-1α increased (WB), the PI3K/Akt signaling activated (WB), the percentage of apoptotic cells significantly reduced after treatment with 1-2% ISO for 6 h (flow cytometry)	[98]
DNP/0.25 mM*	20 min*	Coculture rBM-MSC CM	The viability significantly increased (PKH26, flow cytometry)	[94]

*20 minutes of treatment with 0.25 mM and then reoxidation either 2 or 24 hours in 21% O_2; d: day/days; h: human; m: mouse; r: rat; SD: serum deprivation; CM: cardiomyocytes; HUVEC: human umbilical vein endothelial cells.

(3) Spheroids. Using a spheroid with short-term hypoxia in 1% O_2 poses an advantage over transplantation of individual cells. Spheroids better mimic cellular behavior in native tissue, improving viability, angiogenesis, and immunomodulatory properties [88]. Moreover, interactions of MSCs with endogenous ECM within spheroids increase proliferation and maintain osteogenic differentiation potential influencing bone tissue repair. The synergy of MSC priming with hypoxia and MSC spheroid transplantation is believed to be a good cellular therapy due to increased survival, angiogenic potential, and bone formation. Moreover, spheroids enhance interaction with ECM and promote osteogenesis. Thus, MSC priming under hypoxia and spheroids grafting can be effective in regenerative medicine [89].

3.2.2. Pharmacological and Chemical Hypoxia-Mimetic Agents. Among commercially available pharmaceutical/chemical hypoxia-mimetic agents, the following are discussed below: DFO, DMOG, DNP, $CoCl_2$, and ISO.

DFO is a chelating agent used to remove an excess of iron or aluminum from the body [90]. DFO stabilizes HIF-1α under normoxia; thus, it is a suitable hypoxia-mimetic agent [91]. DMOG is a prolyl hydroxylase inhibitor. DMOG regulates HIF-1α and phosphorylation under hypoxia. DMOG acts via inhibition of factor inhibiting HIF-1α (FIH-1) and the prolyl hydroxylases via competitive inhibition of 2-oxoglutarate (2-OG). It indicates that DMOG can be an effective drug for diabetes due to HIF-1α regulation [92, 93]. DNP increases oxygen consumption due to the enhancement of oxidative metabolism [94]. $CoCl_2$ artificially induces hypoxia and can block the degradation of HIF-1α protein, thus inducing its accumulation [52, 95–97]. ISO is a volatile anesthetic agent. Because of its cytoprotective capacities, it is a good candidate to be a hypoxia-mimetic agent that activates HIF-1α [98].

(1) Cytotoxicity. Table 3 presents the results on the MSC viability upon pharmacologically- or chemically induced hypoxia.

Most studies have been carried out with DFO. It was used in a concentration range of 0.1-500 μm. DFO did not impair the viability of MSCs until 120 μM [97, 99, 100]. The standard preconditioning protocol of MSC treatment with DFO (48 h at a concentration of 3 μM) can be substituted with treatment for 12 hours at a concentration of 50 μM [99]. Fujisawa et al. showed significant cytotoxicity of DFO at a concentration of 10 μM towards BM-MSCs but only after long-term treatment of 53 days [61].

The viability of BM-, UC-, AD-, and DP-MSCs was preserved when $CoCl_2$ was used for 24-48 hours at a concentration of 100 μM [101]. $CoCl_2$ at a concentration of 500 μM significantly decreased MSC viability [102]. DMOG is noncytotoxic until it reaches a concentration of 5 mM [103]. DMOG also increased the proliferation of cocultured cell BM-MSC and human umbilical vein endothelial cells (HUVEC) [52]. ISO increased hBM-MSC metabolism at a concentration of 2% and incubation time of 4 h [98]. DNP at a concentration of 0.25 mM did not injure rBM-MSCs in the coculture with cardiomyocytes, but the treatment period was very short (20 min). Otherwise, this compound could be highly toxic. The cells were slightly shrunken but regained normal morphology after their reoxidation for 2-24 hours. Thus, these results imply that the differences in culture protocols and compound concentrations may be crucial for successfully implementing hypoxia and hypoxia-mimetic agents in regenerative medicine.

(2) Metabolome. The metabolic changes occur in the cells upon adaptation to hypoxia. Metabolome analysis revealed that both hypoxia treatment and DFO administration influence cellular metabolism.

MSCs exhibited metabolic changes in Krebs tricarboxylic acid (TCA) cycle, amino acids, creatine, uric acid, and purine and pyrimidine metabolism upon both types of treatment. DFO-derived hypoxia affected TCA cycle-related metabolism by increasing aconitate, alpha-ketoglutarate (α-KG), and citrate concentrations and decreasing malate and fumarate via reductive carboxylation in reverse Krebs cycle. These effects were more visible for DFO-induced than natural hypoxia (increase only in the α-KG level) [61]. α-KG provides energy for the cellular oxidation of nutrients. The increased α-KG level is required during enhanced cell proliferation. As a precursor of glutamate and glutamine, α-KG acts as an antioxidant agent and directly reacts with hydrogen peroxide. DFO stronger upregulated *α-KG* in comparison to hypoxia, providing better protection against ROS [104].

The low level of malate and fumarate during hypoxia had a positive effect on cells. In contrast, high levels of these compounds were harmful and led to cancer development (by mediating chronic proliferative signals) [105, 106].

The impairment of purine and pyrimidine metabolism is also detrimental to cells, and elevated uric acid levels generated from the purines' metabolism may be responsible for human diseases such as vascular inflammation, atherosclerosis, articular, and gout degenerative disorders [107]. Since phosphoribosyl pyrophosphate (PRPP) is an enzyme involved in synthesizing purine and pyrimidine nucleotides, its level raised under DFO-derived hypoxia [61, 108].

Additionally, 1% hypoxia upsurges the level of the 1-methyl adenosine, a stress marker, compared to DFO-primed MSCs [61, 109]. Further detailed investigations on this topic are required [61, 110, 111].

To summarize, DFO-induced hypoxia affects minor MSC metabolic changes compared to hypoxia. Up to now, detailed metabolome studies have been done only for DFO. Metabolome studies of other hypoxia-mimetic agents are needed to understand the mechanism of their actions and possible short- and long-term side effects.

(3) MSC's Gene Expression. All hypoxia-mimetic agents discussed here increase the expression of HIF-1α, the central controller of adaptive cellular response to hypoxia, and enhance glycolysis similarly to hypoxia [4, 53, 61, 98, 99]. DFO upregulates the genes related to glycolysis: hexokinase 2 (*HK2*), *PDK-1*, BCL-2 interacting protein 3 (*BNIP3*), and

Table 4: Effect of hypoxia pre/treatment on MSC differentiation.

Treatment conditions		Type of stem cells	The effect compared to normoxia (methods of analysis)	Ref.
O_2 concentration	Time/passage			
1%	Pretreatment for 2 d	rBM-MSC	Osteogenesis increased (ALP activity, 7 d; Alizarin Red S, 21 d)	[80]
1%	Pretreatment for 3 d	hBM-MSC	Osteogenic potential of MSC maintained, for high-density spheroid osteogenic potential enhanced; increase in the ALP activity related to the spheroid cell density (ALP staining after 14 d), osteocalcin level maintained (ELISA)	[89]
1%	Pretreatment for 14 d	hBM-MSC	Adipogenic differentiation impaired (Oil Red staining, 14-20 d), osteogenic potential reduced (Alizarin Red staining, 21 d, calcium precipitates detected)	[58]
1%	Pretreatment for 21 d	hAD-MSC	Chondrogenic potential increased (Alcian Blue staining, 21 d); high expression of the *SOX9* and *COL2A1* genes (RT-PCR), osteogenic potential slightly reduced (Von Kossa staining, 21 d, manual counting of calcified areas); lower expression of *ALP* and unchanged *OPN* (RT-PCR), adipogenic potential slightly reduced (Oil Red staining, 21 d); lower expression of the *ADPN* and *LPL* genes (RT-PCR)	[62]
1%	Treatment for 9 d	Coculture hBM-MSC HUVEC	Osteogenic potential maintained (Alizarin Red stain, 9 d), expression of the osteogenic *RUNX2* and *ALP* genes retained and upregulation *COLIA1* (qRT-PCR)	[52]
1.5%	Pretreatment for 1 d	BM-MSC UCB-MSC	Osteogenic potential (Von Kossa staining, 14 d) and expression of the *RUNX2* gene retained (RT-PCR), adipogenic differentiation potential retained (Oil Red staining, 21Coll type IId) and its corresponding marker gene *PPAR-α* (RT-PCR), chondrogenesis (Coll type II detection, 21d), and expression of the *SOX9* gene increased (RT-PCR)	[82]
2%	Pretreatment for 7 d	hAD-MSC	Osteogenesis enhanced (Von Kossa staining, 22Coll type II d), adipogenesis enhanced (Oil Red staining, 21Coll type II d)	[114]
2%	Pretreatment for 8 d	mAD-MSC	Early chondrogenesis increased (Alcian Blue staining, sGAGs assay kit, 6 d), osteogenesis after 7 d maintained (ALP activity, colorimetric assay), after 21 d—decreased (Alizarin Red staining)	[116]
2%	Pretreatment at passage 2 and/or treatment for 21 d	hBM-MSC	Osteogenic potential reduced (Alizarin Red staining, 21 d), osteogenic potential reduced due to hypoxia pretreatment in cells grown in normoxia and hypoxia conditions	[71]
2%	Treatment for 14 d	hBM-MSC	Osteogenic potential maintained (Von Kossa staining and ALP activity, 14 d), adipogenic potential maintained (Oil Red staining, 14 d)	[63]
2%	Treatment for 14 d	hBM-MSC	Osteogenic potential preserved (Von Kossa staining, 14 d), the above capacities preserved up to the seventh passage	[38]
2%	Treatment for 14-17 d	hWJ-MSC	Chondrogenic potential maintained (Alcian Blue staining, 14-17 d)	[64]
2%	Treatment for 18 days	hWJ-MSC	Adipogenic potential increased (Oil Red staining, 18 d)	[63]
2%	Treatment for 21 days	hBM-MSC	Adipogenic potential preserved (Nile Red staining, 21 d) up to the seventh passage	[38]
2%	Treatment for 21 days	hWJ-MSC	Osteogenic potential increased (Von Kossa staining, 21 d)	[64]
2.5%*	Pretreatment for more than 3 days*	hUC-MSC	Osteogenic (Alizarin Red staining, 21 d), adipogenic (Oil Red staining, 21 d), and chondrogenic (Alcian Blue staining, 21 d) potential preserved	[84]
3%	Pretreatment for 7-10 d and/or treatment for 21 d	hBM-MSC	Both hypoxia pretreatment and hypoxic treatment during differentiation preserve osteogenic (Alizarin Red staining, 21 d) and adipogenic (Oil Red staining, 21 d) potential for primary cells only, diminished adipogenesis and inhibited osteogenesis with increase in the passage number from 1 to 4, the genes *ALPL*, *IBSP*, *FABP4*, and *LPL* downregulated (qRT-PCR) with increase in the passage number from 1 to 4, osteogenic differentiation (passage 1) stimulated upon transition from 3 to 20% O_2	[33]

Table 4: Continued.

Treatment conditions		Type of stem cells	The effect compared to normoxia (methods of analysis)	Ref.
O_2 concentration	Time/passage			
1-3%	Pretreatment for 16 h	hBM-MSC	Osteogenic (Alizarin Red staining), adipogenic (Oil Red staining), and chondrogenic (Alcian Blue staining) differential potential maintained	[81]
1-5%	Treatment for 21 d	hBM-MSC	Osteogenic differentiation reduced (Alizarin Red assay, 7, 14, and 21 d), ALP and OPN expressed at low levels below 5% O_2 (WB)	[125]
5%	Pretreatment at passages 2-4 and/or treatment for 21 d	hWJ-MSC	Osteogenesis (Von Kossa staining, 21 d) and cartilage differentiation (Masson's trichrome staining, 21 d) maintained at the same level, hypoxic/normoxic pretreatment and treatment did not affect MSC differentiation potential	[126]
5%	Pretreatment up to passage 2 and/or treatment for 28 d	hBM-MSC	Osteogenic potential (Alizarin Red staining, 28 d), and the expression of the *ALPL* and *RUNX2* genes increased (RT-PCR), adipogenic potential (Oil Red staining, 28 d) increased; the expression level of the *LPL* and *PPARγ* genes maintained (RT-PCR), both hypoxia pretreatment and hypoxic treatment increased differentiation potential	[66]
5%	Treatment for 14 d	hBM-MSC	Osteogenic potential (ALP activity, 14 d) and the *BGLAP*, *RUNX2*, and *COLL2* gene expression increased (RT-PCR)	[65]
5%	Treatment for 21 d	hBM-MSC	Adipogenesis reduced (Oil Red staining, 21 d)	[65]
5%	Treatment for 28 d	hBM-MSC	Chondrogenesis reduced (Alcian Blue staining, 28 d)	[65]
8%	Treatment for 8 d	mBM-MSC	Adipogenesis increased after 8 d (Sudan Black staining)	[123]
8%	Treatment for 14 d	mBM-MSC	*Oct4* inhibited (qRT-PCR)	[123]

*Hypoxic preconditioning in 2.5% O_2 for 15 minutes, then reoxygenation at 21% O_2 for 30 minutes, and again hypoxia preconditioning at 2.5% O_2 for 3 days; d: day/days; h: human; m: mouse; r: rat.

Table 5: Effect of pharmaceutically and chemically derived hypoxia pretreatment on MSC differentiation.

Treatment conditions		Stem cell type	The effect compared to normoxia (methods of analysis)	Ref
An agent/ concentration	Time			
DFO/3 μM	Treatment for 14 d	hBM-MSC	Osteogenic (Alizarin Red staining, 14 d) and adipogenic (Oil Red staining, 14 d) potential decreased	[61]
DFO/15 μM	Treatment for 7 d	hBM-MSC	ALP increased (WB, 7 d)	[120]
DFO/15 μM	Treatment for 21 d	hBM-MSC	Osteogenic potential increased (Alizarin Red staining, 21 d), *ALP*, *RUNX2*, and *OC* upregulated (qRT-PCR), osteogenesis through β-catenin signaling increased (WB)	[120]
DFO/50 μM	Treatment for 21 d	hBM-MSC	Chondrogenesis (Alcian Blue, 21 d) and *SOX9* expression (RT-PCR, 7 d) slightly increased	[124]
DFO/120 μM	Treatment for 8 d	mBM-MSC	Adipogenic potential preserved (Sudan Black staining, 8 d), *Oct4* expression maintained (qRT-PCR)	[123]
DFO/120 μM	Treatment for 21 d	hUC-MSC	Osteogenic (Von Kossa staining, 21 d), and adipogenic (Oil Red staining, 21 d) potential preserved	[97]
$CoCl_2$/100 μM	Pretreatment for 1-2 d	mC3H/ 10T1/2-MSC	Osteogenesis (Alizarin Red staining, 18 d) and expression of the *Col I*, *Bglap*, and *Alp* genes (RT-PCR, 10 d) enhanced, adipogenesis (Oil Red staining, 5 d) and expression of the *Ap2*, *C/ebp*α, and *Ppar*γ genes (RT-PCR, 5 d) reduced, chondrogenesis (Alcian Blue staining, 14 d) and expression of *SOX9*, *Col II*, and *ACAN* genes (RT-PCR, 10 d) enhanced	[101]
$CoCl_2$/100 μM	Pretreatment for 2 d	hDP-MSC hUC-MSC hAD-MSC	Chondrogenesis (Alcian Blue, 28 d) in AD- and UC-MSC increased, in DP-MSC—not detected, cell-source dependent changes of the expression of the following genes: *COL2A1*, *ACAN*, *SOX9*, and *VCAN* (RT-PCR): DP-MSC–*SOX9* (after 7 d up, and after 14, 21, and 28 d downregulated), *VCAN* (after 7, 14, 21, and 28 d upregulated), *COL2A1* and *ACAN* not detected UC-MSC–*SOX9* (after 7, 28 d down, and after 14 and 21 d upregulated), *COL2A1*, *ACAN*, and *VCAN* (after 7,14, 21, and 28 d—upregulated) AD-MSC–*SOX9* (after 7 d up-, and after 14, 21, and 28 d downregulated), *COL2A1* (after 7,14, 21, and 28 d upregulated), and *ACAN* upregulated only after 14 d, *VCAN* (after 7,14, 21, and 28 d downregulated)	[53]
$CoCl_2$/100 μM	Treatment for 8 d	mBM-MSC	Adipogenic potential preserved (Sudan Black staining, 8 d), *Oct4* expression maintained (qRT-PCR)	[123]
$CoCl_2$/100 μM	Treatment for 9 d	Coculture hBM-MSC HUVEC	Osteogenic potential retained (Alizarin Red, nine days), expression of *RUNX2*, *ALP*, *and COLIA1* maintained (qRT-PCR)	[52]
$CoCl_2$/100 μM	Treatment for 21 d	hUC-MSC	Osteogenic (Von Kossa staining, 21 d), and adipogenic (Oil Red staining, 21 d) potential preserved	[97]
$CoCl_2$/100 μM	Treatment for 21 d	hBM-MSC	Chondrogenesis (Alcian Blue, 21 d) and *SOX9* expression (RT-PCR 7d) maintained	[124]
DMOG/ 200 μM	Treatment for 21 d	hBM-MSC	Chondrogenesis (Alcian Blue, 21 d) and *SOX9* and *RUNX2* expression (RT-PCR, 7 d) increased	[124]
DMOG/ 500 μM	Pretreatment for 2 d	rBM-MSC	Osteogenesis (Alizarin Red S, 21 d) and ALP activity (7 d) increased	[80]
DMOG/ 500 μM +1%O_2	Pretreatment for 2 d	rBM-MSC	Osteogenesis (Alizarin Red S, 21 d) and ALP activity (7 d) increased	[80]
DMOG/ 500 μM	Treatment for 9 d	Coculture hBM-MSC HUVEC	Osteogenic potential retained (Alizarin Red, 9 d), expression of *RUNX2* maintained (qRT-PCR), expression of *ALP* and *COLIA1* increased (qRT-PCR)	[52]

d: day/days; h: human; m: mouse; r: rat.

LDHA [61]. DFO upregulates *NUPR* and *p16* expression, improving cell survival [99]. It also induces an increase in the level of HIF-1α by 50-110% while DMOG elevates HIF-1α level by 2-3 times, which is less than $CoCl_2$ stimulating HIF-1α by 2-5 times compared to normoxia. ISO demonstrated the highest impact on the HIF-1α expression (a 150-400% increase). Moreover, DMOG via increasing of HIF-1α expression and activation of the phosphoinositide 3-kinases/protein kinase (PI3K/Akt) signaling pathways regulates cell survival and apoptosis [103]. DMOG lowers

Table 6: Effect of hypoxia on MSC engraftment, migration, and secretion profile.

Treatment conditions		Stem cell type	The effect compared to normoxia (methods of analysis)	Ref.
O_2 concentration	Time/ passage			
1%	1 d	hBM-MSC	*CX3CR1*and *CXCR4* upregulated (qRT-PCR)	[172]
1%	2 d	hBM-MSC	VEGF secretion in spheroids increased (ELISA) on a rat model, collagen deposition (Masson's trichrome stain) enhanced, vascularization and bone formation promoted (high-resolution radiographs), and healing after transplantation of primed MSC spheroids improved compared to transplantation of individual cells	[89]
1%	2 d	hBM-MSC	*VEGF* and *NANOG* upregulated (qRT-PCR)	[57]
1%	2 d 2 d	rBM-MSC	*VEGF* upregulated (RT-PCR), VEGF increased (WB, ELISA)	[80]
1%∗	>2 d*	mBM-MSC	*Cxcr4 downregulated (qRT-PCR)*	[143]
1%	10 d	hBM-MSC	*VEGF* and *NANOG* upregulated (qRT-PCR)	[57]
1%	14 d	mBM-MSC	On a myocardium infarction (MI) mouse model, cardiomyocyte survival reduced due to MCT-4 (WB) increase, and fibrosis in cardiac tissue initiated	[68]
1-3%	16 h	hBM-MSC	Migration potential increased (scratch test)	[81]
2%	Up to 7 passages	hBM-MSC	ECM secretion enhanced (fibronectin and collagen type II fluorescent staining, CLSM), expression of connexin-43 increased (fluorescent staining, CLSM)	[38]
2.5%**	>3 d**	hUCB-MSC	Migration potential increased	[60]
5%***	>8 h***	mBM-MSC	CXCR4, MMP 9, and 14 increased (WB), after MI treatment on the rat model the left ventricular (LV) fibrosis reduced, improved LV function	[152]
5%	4 d	hBM-MSC	VEGF increased (ELISA)	[65]
5%	10 d	hBM-MSC	*MMP7-16* and *TIMP1-3* upregulated (qRT-PCR)	[65]

*Hypoxic pretreatment 4-48 hours at 1% O_2 and then reoxidation 8 hours at 21% O_2; **hypoxic preconditioning in 2.5% O_2 for 15 minutes, then reoxygenation at 21% O_2 for 30 minutes, and again hypoxia preconditioning at 2.5% O_2 for 3 days; ***hypoxic pretreatment 8 hours at 5% O_2 and then 30 minutes of reoxidation at 21% O_2; h: human; m: mouse; r: rat.

myocardial apoptosis [112] via the PI3K/Akt pathway activation. Stabilization of HIF-1α and activation of the PI3K/Akt pathway are crucial for VEGF upregulation.

3.3. Differentiation. This subchapter presents the effects of hypoxia and pharmaceutical/chemical hypoxia-mimetic factors on MSC differentiation. The ability to the multidirectional differentiation is a crucial hallmark of MSC. Furthermore, the differentiation potential and proliferation rate of MSC depend on the type of cells source.

3.3.1. Hypoxia. As described above, stem cells adapt metabolically to hypoxia *in vitro* [113]. The question is whether they differentiate equally efficiently in hypoxia compared to normoxia. The cells can be grown under hypoxia before induction of the differentiation process by the appropriate media (a pretreatment), or lower oxygen tension may be maintained in cultures during differentiation (a treatment). In Table 4, we summarize the available data on the influence of hypoxia on the fate of MSCs cultured in the growth or differentiation media.

Hypoxia pretreatment and treatment can maintain or reduce MSC's osteogenic potential. These effects were observed at the oxygen concentration ranging from 1 to 5% for BM-, AD-, UCB-, UC-, and WJ-MSCs. It may be related to the low expression of the *ALP* and *ALPL* genes coding for *alkaline phosphatases* and the *IBSP* gene coding for an integrin-binding sialoprotein in AD- and BM-MSCs. However, Boyette et al. noted increased *BGLAP*, *RUNX2*, and *COLL2* in hBM-MSC [65].

Hypoxia pretreatment and differentiation in low oxygen conditions (1-5% O_2) preserve BM-, AD-, UCB-, UC-, and WJ-MSC capability for adipogenic differentiation [84, 114]. In BM-, AD-, UCB-, UC-, and WJ-MSCs, the expression of the following adipogenic marker genes, lipoprotein lipase (*LPL*), *PPARα*, peroxisome proliferator-activated receptors (*PPARγ*), and fatty acid-binding protein 4 (*FABP4*), was preserved or even increased.

Nevertheless, inconclusive observations concern the ability to differentiate into cartilage. Chondrogenic potential might be elevated under hypoxia pretreatment [62, 81, 82, 84, 116] and maintained or reduced during hypoxic

Table 7: Effect of pharmaceutically and chemically derived hypoxia on MSC engraftment, migration, and secretion profile.

Treatment conditions		Stem cell type	The effect compared to normoxia (methods of analysis)	Ref
An agent/ concentration	Time			
DFO/10 μM	2 days	hBM-MSC	*VEGF* upregulated (RT-PCR)	[61]
DFO/50-300 μM	1 day	hAD-MSC	*VEGF* increased, the higher DFO concentration induced the higher *VEGF* expression (qPCR)	[102]
DFO/60-600 μM	20 h	hBM-MSC	*CX3CR1*and *CXCR4* upregulated (RT-PCR), CX3CR1 and CXCR4 increased (WB)	[172]
DFO/100 μM	1-3 days	rBM-MSC	*Cxcr4* upregulated (RT-PCR), homing capacities in a NIHL rat model enhanced via PI3K/AKT signal transduction pathway (WB)	[100]
DFO/100 μM	2 days	hWJ-MSC	*VEGF* upregulated (qRT-PCR), mobilization and homing capacities increased	[115]
DFO/150 μM	1 day	hAD-MSC	VEGF increased (ELISA)	[102]
$CoCl_2$/50-300 μM	1 day	hAD-MSC	*VEGF* increased, the higher $CoCl_2$ concentration the higher *VEGF* expression in the range of 50-150 μM, at 300 μM slightly dropped compared to *VEGF* expression at 150 μM (qPCR)	[102]
DMOG/ 500 μM	1 day	hBM-MSC	VEGF increased (WB), angiogenesis increased (tube formation test in the Matrigel), engraftment ability improved, cardiac function improved (left ventricular ejection fraction evaluation), rat model of MI	[92]
DMOG/ 500 μM +1%O_2	2 days	rBM-MSC	*VEGF* upregulated (RT-PCR), VEGF increased (WB, ELISA), angiogenic capability increased in vitro and *in vivo* (tube formation test, Matrigel, rat bone defect model)	[80]
DNP/0.25 mM	20 min	rBM-MSC	The cardiomyogenic genes (*Anp*, *Gata-4*, *Nkx2.5*, *Vegf*, and *Con43*) upregulated (RT-PCR); improvement in cardiac function and significant reduction in scar formation in the rat model of MI	[159]
DNP*/0.25 mM	20 min*	Coculture rBM-MSC CM	*Igf*, *Hgf*, *Vegf*, *Il-7*, *and Il-7r* upregulated (RT-PCR)	[94]
ISO/2%	4 h	hBM-MSC	CXCR4 increased (WB), cell migration increased (hematoxylin and eosin staining, cell count) on a rat stroke model, engraftment and recovery improved	[98]

*20 minutes of treatment with 0.25 mM and then reoxidation either 2 hours or 1 day in 21% O_2. h: human; r: rat.

differentiation in WJ- and BM-MSC in 1-2% O_2 [64, 65]. The expression of chondrogenic marker genes SRY-box transcription factor 9 (*SOX9*) and collagen type II alpha 1 chain (*COL2A1*) followed the above pattern in AD-, BM-, and UCB-MSCs.

Hypoxia pretreatment/treatment influences the MSC differentiation process with effectivity related to passage numbers. In primary cell lines and at the low number of passages, MSCs maintain their differentiation potential compared to the cells passaged many times in the *in vitro* culture under hypoxia [31, 66, 116]. The downregulation of the *FABP4*, *LPL*, *ALPL*, and *IBSP* genes accompanied this diminished capacity of MSCs.

Moreover, individual stem cells under hypoxia are characterized by the enhanced level of plasticity-dependent marker genes such as *NANOG*, *REX-1*, or *Oct4* [117]. The increase in osteogenic potential of individual MSCs was observed compared to monolayer cell culture under normoxia [118]. Oct4 is an essential transcription factor for self-renewal, and it is present in MSCs at low levels on each passage (the higher passage number, the lower Oct4 level). Improved stemness due to higher expression of *Oct4* can result in increased differentiation potential of hypoxia primed stem cells [89, 119].

3.3.2. Pharmacological and Chemical Hypoxia-Mimetic Agents. According to Table 5, the DFO-derived hypoxia treatment during differentiation preserves osteogenic potential and the level of its corresponding marker genes *ALP* and Runt-related transcription factor 2 (*RUNX2*).

DFO treatment maintains or reduces adipogenic potential while increasing chondrogenesis and the expression of *SOX9*. These effects were observed in BM- and UC-MSC after 14-21 days of treatment [61, 97, 120, 121].

$CoCl_2$-derived hypoxia pretreatment increased osteogenesis and upregulated the *Alp*, *Col1*, and osteocalcin (*Bglap*) genes while treatment during differentiation maintained osteogenic potential and the expression of *RUNX2*, *ALP*, and *COLLI*. These effects were observed on mC3H/10T1/2 MSCs and UC-MSCs for 1-9 days [52, 97, 101]. Murine C3H10T1/2 cells are embryogenic cells with features of mesenchymal stem cells and thus represent interesting research objects. They have the potential to be an attractive alternative source of primary BM-MSCs in studies of

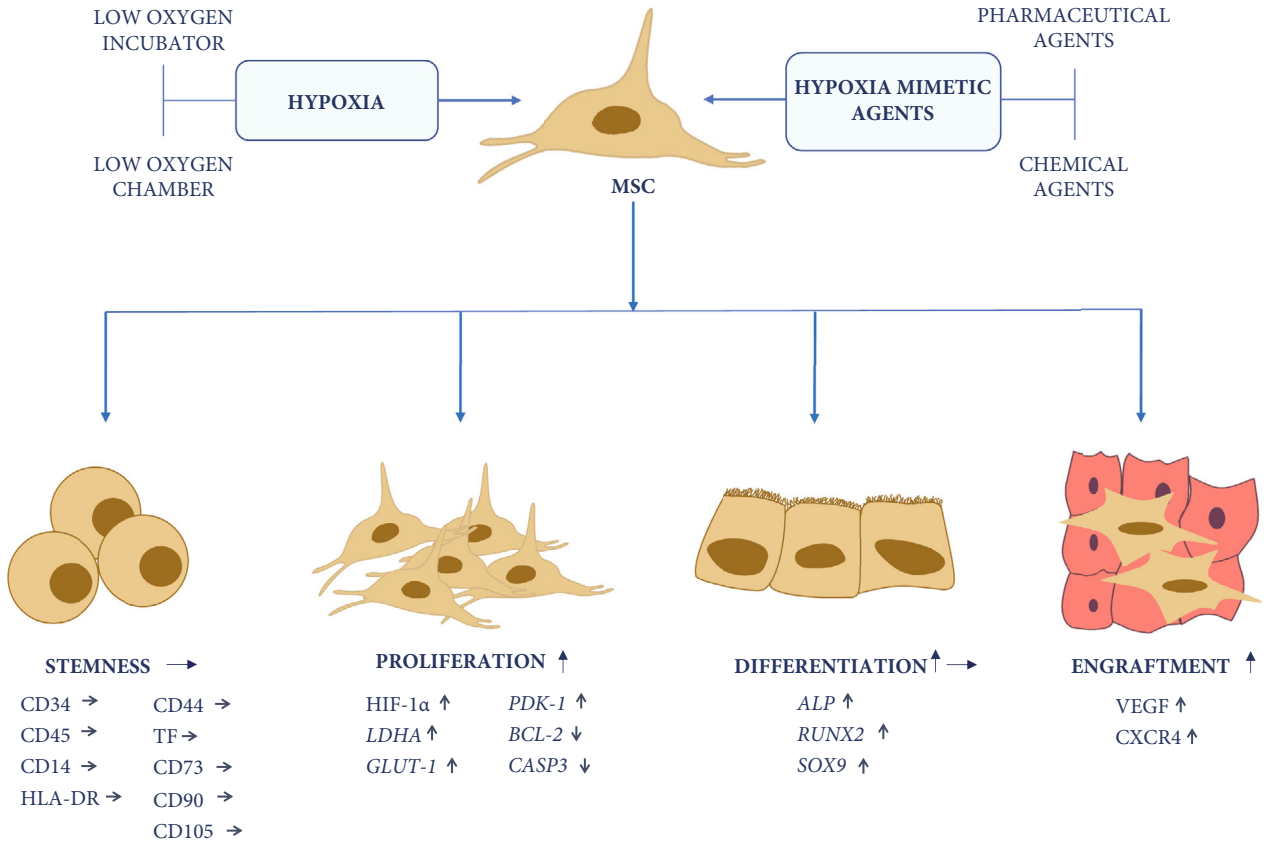

Figure 1: Hypoxic pretreatment methods for the stem cell efficient culture. Both hypoxia and hypoxia-mimetic agents can influence MSC homeostasis preserving their stemness. Only the major proteins and genes related are presented.

osteogenic and chondrogenic differentiation for regenerative medicine [122]. $CoCl_2$-derived hypoxia pretreatment decreases adipogenesis and the marker genes Apetala 2 (*aP2*), CCAAT/enhancer-binding protein α (*C/ebpα*), and *Pparγ* in mC3H/10T1/2 MSC for 24-48 hours. On the opposite, $CoCl_2$-derived hypoxia treatment maintained adipogenicity in BM-MSCs for eight days [101, 123]. $CoCl_2$-derived hypoxia pretreatment and treatment increase chondrogenesis and the expression of the chondrogenic marker genes *SOX9*, *Coll2a1*, *VCAN*, and aggrecan (*ACAN*) in mC3H/10T1/2, BM-, UC-, AD-, and DP-MSCs for 2-21 days [53, 101, 124].

DMOG-derived hypoxia treatment of BM-MSCs maintained osteogenesis and *RUNX2* expression and upregulated *ALP* and *COLLIA1* for nine days [52] as well as chondrogenesis and *SOX9* marker for 21 days [124].

The bone fracture niche is hypoxic; therefore, oxygen tension is critical in bone healing. Nguyen et al. performed a direct coculture of BM-MSCs and HUVEC in normoxia and the chemically activated hypoxia with $CoCl_2$ and DMOG. Under hypoxia induced by $CoCl_2$, von Hippel–Lindau protein (pVHL) which binds to the oxygen-degrading domain and prevents hydroxylation of HIF-1α by oxygen-dependent prolyl hydroxylases (PHD) was inhibited [127]. DMOG can directly inhibit PHD and stabilize HIF-1α[124, 128] compared to normoxia. Normoxia generally promotes bone formation in MSCs and HUVEC coculture, while hypoxia favors angiogenesis. DMOG is a more promising hypoxia-priming agent than $CoCl_2$ because it stronger enhances endothelial marker—von Willebrand factor (VWF) and VEGF [129]. Moreover, coculture (in the ratio 1 : 1) of BM-MSCs and HUVEC promotes osteogenesis in MSCs under normoxia, and hypoxia even enhances this effect [52].

Unfortunately, up to date, no data on the influence of DNP or ISO on MSC differentiation are available in the literature. Concerning the published results, it may be assumed that the effect of pharmaceutical/chemical hypoxia-mimetic agents on MSC differentiation is similar to hypoxia. However, the period of this enhancement has not been studied yet. The currently available scientific data also do not allow concluding whether hypoxia-inducing chemical agents could efficiently reduce the time required for cell differentiation.

3.4. Engraftment, Migration, and Secretion Profile. Successful MSC engraftment is crucial in regenerative medicine. The high proliferation rate and prominent expression of chemokine receptors on MSCs are attributed to young cells providing migration and potential therapeutic increase after transplantation [45].

The latest data indicate that chemokines and their receptors are critical in migration, chemotaxis, and homing *in vitro* and *in vivo* [130]. There are different BM-MSC-

related chemokine receptors, such as CXC, but insufficient data are available on their function in cell therapy [131]. The rat brain ischemia model shows that chemokines C–C motif *chemokine* ligand 2 (CCL25) and C-X3-C motif chemokine ligand 1 (CX3CL1) can also influence MSC chemotaxis [131]. Moreover, CC-type chemokines are involved in cellular implantation and remodeling following transplantation [130].

HIF-1α causes upregulation of chemokine receptors on MSCs [132]. Under hypoxic conditions, the stabilized HIF-1α is shifted into the nucleus to bind the HIF-1β forming heterodimer. Subsequently, the heterodimer attaches to hypoxia response elements (HREs) linked with CREB-binding *protein*/p300 protein (CBP/p300) [133, 134] and increases the expression of chemokine receptors C-X3-C motif chemokine receptor 1 (CX3CR1), C-X-C chemokine receptor type 7 (CXCR7), and C-X-C motif chemokine receptor 4 (CXCR4). Hypoxia can increase *CXCR4* expression [135]. Hypoxia-induced upregulation of *CXCR4* may result from HIF-1α stabilization [136]. Metabolic flexibility is one of the features represented by MSCs, helping them survive under ischemic stress and maintaining their multipotency [137]. HIF-1α is one of the master regulators controlling the cellular response to the tension caused by low oxygen levels [138].

HIF-1α is also involved in the CXCR4 expression induced by the activation of HREs in the Ets1 promoter, a transcription factor of CXCR4. Changes in the oxygen level are an essential regulator of CXCR4 expression. Hypoxia stabilizes CXCR4 transcripts, contributing to an increase in the *CXCR* gene expression. It suggests that hypoxia-regulated RNA binding proteins could influence CXCR4 stabilizing its mRNA at the posttranscriptional level [139].

Angiogenesis is vital in tissue engineering because of tissue blood flow restoration and new blood vessel formations [140]. Proangiogenic factors (VEGF and matrix metalloproteinases (MMPs)) and antiangiogenic factors (endostatin and tissue inhibitor of metalloproteinases (TIMPs)) are involved in angiogenesis regulation [141]. Applications of proangiogenic proteins in stroke and myocardial infarction treatment have been reported [142].

3.4.1. Hypoxia. As shown in Table 6, hypoxia increases MSC migration via upregulation of chemokine receptors *CXCR1* and *CXCR4*.

This effect was observed in the O_2 concentration ranged from 1 to 5% when BM- and UC-MSC were grown for 8-48 hours [60, 81]. The *CXCR4* gene expression decreased in C57BL/6 murine BM-MSCs exposed to acute hypoxia compared to normoxia. The reduction of the *CXCR4 gene* expression could result from the long-term culture of cells in normoxia followed by acute hypoxia shock. In the next step, MSC reoxygenation after hypoxia led to the *CXCR4* gene expression decreasing. The reduction of the *CXCR4* gene expression during the second stage of reoxygenation could have been caused by the compatibility of cells to new oxygen conditions—hypoxia following the suppressive effect of normoxia on the *CXCR4* promoter [143].

Hypoxia also increases the angiogenic capacity of MSCs. This effect might be observed upon O_2 concentration ranging from 1% to 5% after incubating BM-MSCs for 2-4 days [57, 80, 89]. The *VEGF* gene expression increased under hypoxic conditions [119, 144]. VEGF and Angiopoietin 1 (Ang-1) play a crucial function in angiogenesis, and their increase is essential for successful stem cell transplantation [102, 145]. Decrease of high mobility group box protein 1 (HMGB1) nuclear protein under hypoxia is believed to protect tissue from damage [80]. MSC's spheroids promote vascularization and bone formation [89].

Cell migration, vascularization, and tissue remodeling in bone are MMP/TIMP dependent. The family of TIMP proteins controls MMPs' function. MMP-2, MMP-9, MMP-13, and TIMP-1 are crucial in bone formation and repair [65]. MMP-9 and MMP-13 are involved in the recruitment and activation of osteoclasts [146–148]. MMP-2 is essential for generating spatial osteolytic structures and mineralization [149]. A loss of its function can disrupt proliferation and osteoblastic differentiation, disturbing skeletal development [150], and mutation in the *MMP-2* gene might cause bone diseases [151]. Hypoxic preconditioning showed upregulation of many *MMP* and *TIMP* genes in 5% O_2 up to ten days in BM-MSCs [65, 152]. Long-term hypoxic cultivation upregulates *MMP7-16* and *TIMP1-3* but downregulates *MMP2*. There are few experiments on this topic, but it requires further investigation.

Heart damage is one of the common diseases of modern civilization [153]. Cardiomyocytes, endothelial cells, fibroblasts, and perivascular cells are crucial in heart homeostasis. Transplantations of two cell types, cardiomyocytes (CMs) and vascular cells, exhibited better therapeutic effects in infarcted hearts [154]. Moreover, the coculture of myocytes with endothelial cells enhances myocytes' survival *in vitro*[155]. New, more efficient strategies are still needed. Mathieu et al. noted that hESC could reenter pluripotency under hypoxia conditions, and this dedifferentiation depends on HDAC activity [156]. The iPSC research seems to be very promising, as it does not raise ethical questions such as the hESC [157]. Practical methods for differentiating murine iPSC-derived cardiomyocytes, combining hypoxia and bioreactor controlling culture conditions, have already been described [158]. Extracellular vesicles (EVs) are attracting the attention of researchers because of their ability to mimic all the therapeutic effects induced by the MSCs (e.g., anti-inflammatory, proangiogenic, or antifibrotic) [159]. Thus, MSC-derived EVs can modulate tissue response to a broad spectrum of injuries [160] and are considered a substitute for cell-based therapies. The clinical studies using exosomes in the treatment of cardiovascular disease are at an early stage [161–163]. For example, the exosomes derived from BM-MSCs [161, 163] or umbilical cord- (UC-) MSCs [164] showed the positive influenced cardiac function (preclinical model of MI) [165]. Hypoxia and DFO preconditioning of MSC for EV delivery is the developing strategy for regenerative medicine [166, 167].

3.4.2. Pharmacological and Chemical Hypoxia-Mimetic Agents. As shown in Table 7, pharmaceutical/chemical hypoxia-mimetic agents can improve the migration and angiogenic capabilities of MSCs.

An increase in migration was observed after BM- and WJ-MSC incubation with DFO and ISO for 4-72 hours [98, 100, 115]. The increased expression of VEGF was noted in WJ-, AD, and BM-MSCs after their treatment with DFO, DMOG, or DNP for 20 min and 48 hours [94, 115, 168].

Preconditioning of MSC with DMOG was applied in the harvesting of cells for application in the treatment of heart ischemia [4, 92], cartilage regeneration [124], and bone regeneration in an aged population [45, 80]. DNP has already been used as a hypoxia-mimetic agent on numerous cell types such as neonatal cardiomyocytes, neurons, H9C2, and embryonic cardiac cells [169–171]. Preconditioning of stem cells with DNP improved their adhesion, survival, homing capacities, and cardiomyogenic genes such as *Gata*-binding *protein 4 (Gata-4)*, NKX2 homeobox *5 (Nkx2.5*), Connexin 43 (*Con43*), atrial natriuretic peptide (*Anp*), and *Vegf* [168]. MSC priming with DNP was used in the myocardium regeneration process [94] and improved cardiac function [168]. Similarly, preconditioning of MSCs with ISO improved their migration and engraftment into the ischemic brain (the rat model of stroke) [98].

Hypoxia increases migration and vascularization of MSCs and protects them against apoptosis. It was revealed that pharmaceutical/chemical hypoxia-mimetic agents stronger enhance the expression of chemokine receptors and VEGF than hypoxia. The exact effect depends on the hypoxia-mimicking agent. Moreover, chemokine receptor studies were performed only for DFO and ISO. There is no data about the influence of other hypoxia-derived agents on chemokine expression. Moreover, there was no information about essential proteins and MMP/TIMP changes upon treatment of MSCs with hypoxia-mimetic agents.

4. Conclusions

Clinical applications of MSCs gave insufficient effects due to low survival, retention, or the insufficiency of cell differentiation. Hypoxia conditions mimic the natural tissue environment preserving embryonic development and the pluripotency of stem cells and enhancing angiogenesis. The knowledge on MSC priming is critical in evaluating safety procedures and potential use in clinics. Hypoxia preconditioning *in vitro* uses 2-5% oxygen concentration. It preserves MSC's differentiation potential, upregulates chemokine receptors, and delays cell senescence in a source-dependent manner. There are clear pieces of evidence that both hypoxia pretreatment and treatment are beneficial for MSC differentiation. Hypoxia priming has been proved as a practical approach for ischemic stroke and other disability treatment.

A growing group of pharmaceutical/chemical hypoxia-mimetic agents concur with hypoxia chambers and incubators, acting similarly according to the current knowledge (Figure 1). Pharmaceutical/chemical hypoxia-mimetic agents can also increase cell proliferation, preserve or enhance differentiation potential, increase migration potential, and induce neovascularization in a concentration- and stem cell source-dependent manner. According to the current knowledge, they act via upregulation of HIF-1α, leading to changes in the metabolism, e.g., increasing glycolysis. Pharmaceutical/chemical hypoxia-mimetic agents might find several applications in human medicine. DFO can be used in the general preconditioning of stem cells in regenerative medicine (due to contrary data on osteoblastic differentiation, its application in bone regeneration requires further investigation). $CoCl_2$ is proposed for cartilage regeneration. DMOG has been applied in myocardial infarction, ischemic heart, brain, and bone regeneration in the aged population. Moreover, it is a better candidate for cartilage tissue regeneration compared to DFO and $CoCl_2$. DNP is believed to promote cardiac regeneration, and ISO can be used in ischemic brain treatment.

However, current literature still shows certain contradictory data on the influence of hypoxia on MSC functions. This phenomenon stems from differences in the protocols used, culture conditions, media composition, hypoxia conditions and timing, and the heterogeneity of cell donors. At least on some hypoxia inducers, our knowledge of the mechanisms is not sufficiently comprehensive, affecting their potential use. Up to now, DFO is the most studied agent for MSC priming and seems to be a quite safe choice. Metabolome changes in DFO-derived hypoxia are less harmful to MSCs compared to hypoxia. Many new hypoxia-mimetic agents have not yet been fully characterized. One of these agents is DMOG, which is going to have great potential in MSC preconditioning.

DFO and hypoxia-mimetic agents in optimized treatment conditions can improve MSC lifespan and maintain or increase their differentiation potential, migration, and immunomodulatory properties for successful engraftment in a hypoxia inducer concentration-dependent manner. The optimal culture conditions and pharmaceutical/chemical agent concentration should be optimized for priming stem cells to translate the results from *in vitro* effectiveness to *in vivo* conditions.

To summarize, preconditioning using DFO and other pharmacological/chemical hypoxia-mimetic agents positively affects MSC viability and other properties. They have not been studied so wildly as hypoxia but are believed to find application as pretreatment for many diseases considering their low cost and ease of use.

Abbreviations

AD:	Adipose-derived
α-KG:	Alpha ketoglutarate
ALP:	*Alkaline phosphatase*
ANP:	Atrial natriuretic peptide
aP2:	Adaptor *protein* 2
BAX:	BCL-2-associated X
BCL-2:	B-cell lymphoma 2
bFGF:	Basic fibroblast growth factor
BGLAP:	Bone gamma-carboxyglutamic acid-containing protein (osteocalcin)
BM:	Bone marrow
CASP-3:	Caspase 3
CD:	Crohn's disease
C/EBPα:	CCAAT enhancer binding proteins

CBP/p30: CREB-binding *protein*/p300 protein
CCL25: C–C motif chemokine ligand 25
CMs: Cardiomyocytes
$CoCl_2$: Cobalt chloride
Coll2a1: Collagen alpha-1(II) chain
Cx43: Connexin-43
CX3CL1: C-X3-C motif chemokine ligand 1
CX3CR1: C-X3-C motif chemokine receptor 1
CXCR4: C-X-C motif chemokine receptor 4
CXCR7: C-X-C chemokine receptor type 7
DFO: Deferoxamine
DNP: 2,4-Dinitrophenol
DM: Diabetes mellitus
DMOG: Dimethyloxaloylglycine
ESC: Embryonic stem cell
FABP4: Fatty acid-binding protein 4
Gata-4: *Gata*-binding *protein 4*
GLUT-1: Cellular glucose transporter-1
G6PD: Glucose-6-phosphate dehydrogenase
GVHD: Graft-versus-host disease
hESC: Human embryonic stem cells
HIF-1α: Hypoxia-inducible factor 1 alpha
hiPSC: Human-induced pluripotent stem cells
HK2: Hexokinase 2
HLA-DR: Human leukocyte antigen-DR isotype
HMGB1: High mobility group box protein 1
HREs: Hypoxia response elements
HUVEC: Human umbilical vein endothelial cells
IBSP: Integrin-binding sialoprotein
LDHA: Lactate dehydrogenase A
LPL: Lipoprotein lipase
mC3H/10T1/2: Murine embryonic mesenchymal cell line
MMP-9: Matrix metalloproteinase 9
MS: Multiple sclerosis
MSC: Mesenchymal stem cell
NANOG: Homeobox transcription factor
Nkx2.5: NKX2 homeobox *5*
Notch: Translocation-associated (Drosophila), a *signaling pathway*
ISCT: International Society for Cellular Therapy
ISO: Isoflurane
Oct4: Octamer-binding transcriptional factor 4
2-OG: 2-Oxoglutarate
P53: Cellular tumor antigen
PDK-1: 3-Phosphoinositide-dependent protein kinase 1
PHD: Oxygen-dependent prolyl hydroxylases
PGF: Placental growth factor
PI3K: Phosphoinositide 3-kinases
PRPP: Phosphoribosyl pyrophosphate
pVHL: von Hippel–Lindau protein
REX-1: Reduced expression protein-1
ROS: Reactive oxygen species
RUNX2: Runt-related transcription factor 2
SOX2: SRY-box transcription factor 2
SOX9: SRY-box transcription factor 9
SSEA-3: Stage-specific mouse embryonic antigen-3
SSEA-4: Stage-specific mouse embryonic antigen-4
TCA: Tricarboxylic acid cycle
TIMPs: Tissue inhibitor of metalloproteinases
Tra-1-60: T cell receptor alpha locus 1-60
UC: Umbilical cord
UCB: Umbilical cord blood
WJ: Wharton's jelly
VEGF: Vascular endothelial growth factor
VEFG-D: Vascular endothelial growth factor-D
VCAN: Versican core protein
VWF: von Willebrand factor.

Conflicts of Interest

The authors declared no conflict of interest.

Authors' Contributions

Study design was contributed by A.N-S, literature review was contributed by A.N-S and P.N.O, writing original draft paper was contributed by A.N-S and E.A.T, and writing—review and editing—was contributed by E.A.T. and H.F.

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Review Article

Advances in Regulatory Strategies of Differentiating Stem Cells towards Keratocytes

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Corneal injury is a commonly encountered clinical problem which led to vision loss and impairment that affects millions of people worldwide. Currently, the available treatment in clinical practice is corneal transplantation, which is limited by the accessibility of donors. Corneal tissue engineering appears to be a promising alternative for corneal repair. However, current experimental strategies of corneal tissue engineering are insufficient due to inadequate differentiation of stem cell into keratocytes and thus cannot be applied in clinical practice. In this review, we aim to clarify the role and effectiveness of both biochemical factors, physical regulation, and the combination of both to induce stem cells to differentiate into keratocytes. We will also propose novel perspectives of differentiation strategy that may help to improve the efficiency of corneal tissue engineering.

1. Introduction

In the eye, the cornea is the outermost structure—a highly organized and specialized transparent tissue that plays a vital role in both the refraction of light onto the retina and protecting the eye from infectious agents. When the cornea is injured, activated keratocytes in the corneal stroma will transform into fibroblasts and myofibroblasts and subsequently migrate to the wound for tissue remodeling, including alignment of collagen fibrils. However, under pathophysiological conditions, a fibrotic process may occur, resulting in corneal scar formation, including misaligned collagen fibrils. The pathophysiology of corneal scar formation is still poorly understood, thus the current lack of treatment to restore the structure of the collagen fibrils and regain vision. Corneal transplantation is presently the only available curative treatment for corneal scars, but even in many developed countries, it is difficult to perform transplantations due to lack of donors. Therefore, new therapeutic methods to treat corneal scar formation are warranted.

Recently, much research focus has been on corneal tissue engineering [1–7]. The research has mainly focused on attempts to create artificial corneal constructs to implant clinically in patients, which could potentially solve the problem of limited donors. In these attempts, stem cells are commonly used and differentiated into corneal keratocytes. However, the utilization of stem cells in corneal tissue engineering is not without challenges since the appropriate microenvironment is crucial to achieve differentiation of stem cells specifically into keratocytes.

In this review, we aim to clarify the role and effectiveness of both biochemical factors, physical regulation, and the combination of both to induce stem cells to differentiate into keratocyte. In addition, we will propose novel perspectives of differentiation strategy that may help to improve the efficiency of corneal tissue engineering.

2. Cornea and Corneal Injury

The cornea is comprised of five main layers from front to back: epithelium layer, Browman's membrane, stroma layer, Descemet's membrane, and endothelium layer (Figure 1) [8]. The avascular stroma accounts for 80%-85% of the

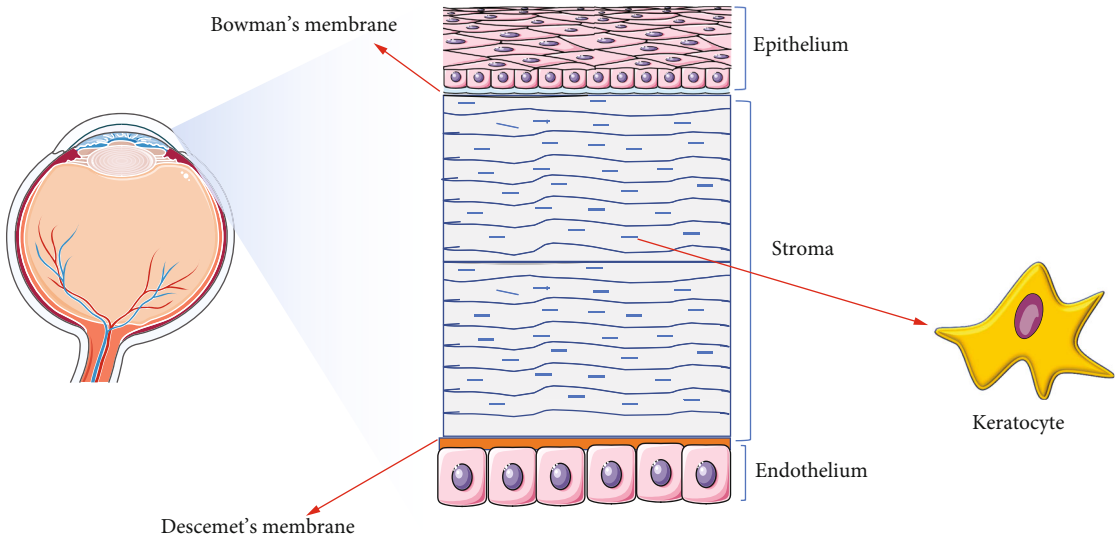

Figure 1: Schematic structure of the cornea. The epithelial layer is the outermost part of the corneal tissue, which sits on Bowman's membrane. The stroma layer is the middle part of the corneal tissue which accounts for 80%-85% of the cornea's thickness and consists of mainly keratocytes. The endothelium layer is the innermost part of the corneal tissue and is connected to the stroma layer by Descemet's membrane.

cornea's thickness, which is around 0.4 mm at the center and gradually increases in thickness towards the periphery [9]. It consists of keratocytes, collagens (mainly collagen type I and type V), glycosaminoglycans (GAGs), and proteoglycans (PGs) [8]. Maintenance of transparency and mechanical characteristics in the stroma is highly dependent on its orthogonal orientation of collagen fibers, arranged into bundles called lamellae, with a thickness of 2 μm-200 μm. In between the lamellae, the sparsely scattered collagen-producing keratocytes are found [10]. The keratocytes are normally considered quiescent and exhibit a dendritic morphology with processes extending and interacting with neighboring cells and express cluster of differentiation 34 (CD34) and aldehyde dehydrogenase 3 family (ALDH3A1). Keratocytes produce various important PGs including keratocan (KERA), lumican (LUM), mimecan, decorin, and biglycan [8]. These PGs consist of different GAGs such as keratan sulfate, chondroitin sulfate, and dermatan sulfate [9]. Furthermore, keratocytes are responsible for the synthesis of collagen molecules and matrix metalloporteases (MMPs) which is of significance in maintaining the homeostasis of corneal stroma [9].

Corneal injury is one of the leading causes of blindness, affecting millions of people worldwide [11]. When the cornea is injured, activated corneal stroma keratocytes will no longer be able to perform its physiological functions but will instead transform into fibroblasts and myofibroblasts that under pathophysiological conditions may result in scar formation and blindness. According to the survey of WHO, there are over half a million people worldwide who are suffering from blindness caused by eye injuries and around 48% of them were specifically caused by corneal injuries [12, 13]. Currently, the main therapeutic option for corneal impairment is corneal transplantation. The demand of corneal transplantation has gradually increased, but there is a lack of accessible cornea for transplantation. It is reported that around 12.7 million individuals are waiting for corneal transplantation but only 1/7 can be transplanted, mainly due to lack of donors [8, 14]. According to a survey done in 2016, the median waiting time was 6.5 months before a suitable cornea was donated for transplantation while in many countries, patients were unable to receive a transplantation and became blind [15]. Therefore, there is an urgent need of new strategies to tackle the shortage of corneal donors for transplantation.

3. Corneal Tissue Engineering

Tissue engineering is an interdisciplinary research field that is aimed at restoring or regenerating the impaired tissues *in vivo*. More specifically, cells are isolated from tissue and expanded in culture *in vitro*. Biomaterials (scaffold) and/or biochemical factors are combined with the cells and subsequently implanted to the body to allow regeneration of the defected tissues or organs to improve the quality of patients' life [16, 17]. Up to now, tissue engineering has already been applied in the regeneration of miscellaneous tissues/organs such as the liver [18–20], spinal cord [19, 21], skin [22–24], cartilage [25], and blood vessels [26]. In the field of corneal tissue engineering, researchers have transplanted decellularized human corneal lamina with autologous adipose-derived MSCs (ASCs) into patients with corneal defects to evaluate its safety, tolerability, and preliminary efficacy. In addition to this, ex vivo cultivated human corneal stromal stem cells (CSSCs) have been transplanted into recruited patients to treat corneal blindness. These clinical trials to achieve regeneration of the human cornea and the restoration of vision have been summarized in Table 1. Since clinical trials show promising results, it is tempting to believe that tissue engineering can be used to replace impaired cornea to tackle the shortage of corneal donors.

Table 1: Clinical trials of stem cells used for corneal regeneration.

ID	Title	Phase	Patients (*n*)	Stem cells	Outcome
NCT02932852	Autologous Adipose-Derived Adult Stem Cell Transplantation for Corneal Diseases	Early phase 1	12	hASCs	Vision recovery; topography; anterior segment optical coherence tomography; slit lamp observation; refraction measurement
NCT03878628	Treatment with Allogeneic Adipose-derived Mesenchymal Stem Cells in Patients with Aqueous Deficient Dry Eye Disease (MESADDE)	Early phase 1	7	hASCs	Injection site: pain, infection, bleeding; eyelid function disorder; periorbital edema; ocular discomfort; flu-like symptoms; fever; Ocular Surface Disease Index questionnaire; Schirmer's *I* test; tear osmolarity; Ocular SICCA Grading Score; HLA antibodies
NCT04932629	To Evaluate the Clinical Safety and Efficacy of Limbal Stem Cell for Treatment of Superficial Corneal Pathologies	Early phase 1	20	hCSSCs	Measurement of any ocular or systemic adverse effects; measurement of visual improvement; change in corneal light scattering
NCT01377311	The Improvement of Limbal Epithelial Culture Technique by Using Collagenase to Isolate Limbal Stem Cells	Phase 1	10	hCSSCs	Using collagenase to isolate limbal stem cells and improve the technique of ex vivo expansion of limbal stem cells for the treatment
NCT03295292	Limbus-derived Stem Cells for Prevention of Postoperative Corneal Haze	Phase 1	15	hCSSCs	Maintenance of preoperative best spectacle-corrected visual acuity; efficacy in reducing corneal light scatter using Scheimpflug imaging
NCT02948023	Stem Cells Therapy for Corneal Blindness (ExCell)	Phase 1	100	hCSSCs	Ocular or systemic adverse effects; visual improvement after treatment
NCT04484402	Treatment of Patients with Inflammatory-dystrophic Diseases of the Cornea Using Autologous Stem Cells	Phase 1 Phase 2	25	hASCs/ hCSSCs	Number of cured patients, patients with treatment-related adverse events
NCT01562002	Safety Study of Stem Cell Transplant to Treat Limbus Insufficiency Syndrome	Phase 1 Phase 2	27	hBMSCs/ hCSSCs	Viability and safety of mesenchymal stem cell transplant; absence of complications in pre- and perisurgical implantation; improvement of 2 lines in best-corrected visual acuity
NCT02148016	Corneal Epithelium Repair and Therapy Using Autologous Limbal Stem Cell Transplantation	Phase 1 Phase 2	30	hCSSCs	Composite measure of visual function in eyes treated for corneal ocular surface disease; composite measure of visual function in eyes after photorefractive keratectomy; incidence of transparency of the cornea; postoperative complications
NCT02592330	Limbal Stem Cell Deficiency (LSCD) Treatment with Cultivated Stem Cell (CALEC) Graft (CALEC)	Phase 1 Phase 2	17	hCSSCs	The occurrence of ocular infection, corneal perforation, graft detachment ≥ 50%, and adverse events and their relationship to the study intervention; obtaining cell growth and maintaining cell viability; avoiding culture contamination; improvement in corneal surface integrity; decrease in neovascularization; decrease in subject symptoms

Table 1: Continued.

ID	Title	Phase	Patients (*n*)	Stem cells	Outcome
NCT02318485	Limbal Epithelial Stem Cell Transplantation: A Phase II Multicenter Trial (MLEC)	Phase 2	60	hCSSCs	Visual acuity; presence of persistent epithelial defects; presence of corneal conjunctivalization; change in corneal vascularization; pain; photophobia; rejection
NCT04615455	Mesenchymal Stem Cell Therapy of Dry Eye Disease in Patients with Sjögren's Syndrome (AMASS)	Phase 2	40	hASCs	OSDI; noninvasive keratography tear break-up time (NIKBUT); tear meniscus height (TMH); Schirmer's *I* test; tear osmolarity; Oxford scale; HLA antibodies

Source of data: all comes from https://clinicaltrials.gov.

4. Cells Used in Tissue Engineering of Corneal Stroma

In recent years, there have been large number of studies on corneal tissue engineering. The selection of appropriate cells is important for successful tissue engineering and is considered the first core factor in the field of tissue engineering. Even though keratocytes are the main cell type in the normal cornea, it is not a suitable cell type for corneal tissue engineering because of the difficulty to culture keratocytes *in vitro*. When keratocytes are cultured in medium containing serum, they quickly lose their dendritic morphology, decrease the expression of specific keratocyte markers, and instead transform into a fibroblast and myofibroblast phenotype [27] in a similar manner as in injured cornea. To prevent the phenotype drift of cultured keratocytes, serum-free medium has been developed. Although keratocyte phenotype can be maintained by culturing them in serum-free medium, their low proliferation rate makes it hard to obtain enough cells required for tissue engineering [27]. Some researchers have generated a cell culture system which can both ensure the expansion of human keratocytes and the preservation of their dendritic morphology by culturing them on human amniotic membrane [27]. However, the procedure is complex and requires expensive growth factors [27, 28]. Therefore, based on the arguments raised above, keratocytes are not suitable cells for corneal stroma tissue engineering.

Stem cells, which obtain outstanding proliferative capacity and the potential to differentiate into keratocytes, have been widely used in corneal stroma tissue engineering. The main types of stem cells used are CSSCs [29–31], embryonic stem cells (ESCs) [5, 32–35], and mesenchymal-derived stem cells (MSCs) [27, 36–38]. MSCs are mainly divided into two categories: bone marrow-derived MSCs (BMSCs) and ASCs. CSSCs could be isolated from the limbal region of human corneas, using the neutral protease dispase. Unlike keratocytes, CSSCs are able to undergo extensive expansion *in vitro* without losing the ability to differentiate into keratocyte phenotype [29]. CSSCs embedded in compressed collagen gel has been injected into the injured cornea of mouse which resulted in successful regeneration without scar formation after 2 weeks [39]. Compared with the CSSCs, MSCs are easier to obtain from either bone marrow (BMSC) or adipose tissues (ASC), and their self-renewal ability has been proven. Liu et al. injected BMSCs into the kera$^{-/-}$ or lum$^{-/-}$ mouse model. It was found that BMSCs were able to survive in the corneal stroma and to differentiate into a keratocyte phenotype [36]. However, MSCs have the potential to differentiate into multiple cell types such as cardiomyocytes and vascular endothelial cells, thus making a specific differentiation into keratocytes a challenge [27, 29]. ESCs, which derived from the inner cell mass of human blastocyst, appear to have an unlimited lifespan and the potential to differentiate into any somatic cell type [32]. However, ESCs need to be cocultured with other cells, such as the mouse fibroblast line PA6, to differentiate into the neural crest-lineage, that is considered to be the origin of keratocytes, before being differentiated into keratocyte phenotype [32]. Yet, the ethical aspect of using ESCs hampers its application in corneal tissue engineering [40]. As a result of a profound exploration of the corneal keratocytes, researchers found that dental stem cells (DSC) and keratocytes share the same origin from the neural crest lineage. In addition, DSCs are easily accessible and share similar proteoglycan secretion profile as keratocytes which make them a promising cell type for corneal tissue engineering [41, 42]. As an example, dental pulp stem cells (DPSCs) injected into mouse corneal stroma was discovered to form a stromal extracellular matrix (ECM) without rejection, thus suggesting that it may serve as a possible choice of cells for corneal regeneration [43]. Although all the above-mentioned stem cells are promising for corneal tissue engineering and regeneration, they have great uncertainty since they harbor multipotent differentiation potential. Therefore, many studies have tried to improve the efficiency of cell differentiation towards keratocytes by using various strategies of regulation.

5. Current Strategies for Inducing Differentiation of Stem Cells into Keratocytes

Extensive studies have shown that the microenvironment plays a significant role in modulating the differentiation fate of stem cells, which includes stem cells in the bone marrow [44], skin [45], intestine [46], brain [47], spinal cords [48], and others [49, 50]. The microenvironment includes both the physical (stiffness, stress or strain relaxation, etc.) and biochemical factors (growth factors or cell adhesion

molecules, etc.) [51]. As an example, in osteogenesis, it was found that both the cell-cell contact (transmission of growth factors, i.e., biomechanical factors) and the substrate stiffness (physical environment) played important roles in the differentiation of MSCs towards osteoblasts and are therefore considered important in regeneration of bone [52]. The sophisticated 3D ECM of corneal stroma consists of highly aligned collagen layers and multiple growth factors including insulin-like growth factors (IGF), transforming growth factor beta (TGF-β), and fibroblast growth fator-2 (FGF-2) [53, 54]. The cells in the corneal stroma also receive various kinds of physical stimulations (the stiffness of ECM, local topography, and stress), which is crucial for the developmental morphogenesis, reaction to fluctuating intraocular pressure, and wound healing process of keratocytes [55].

Inspired by the microenvironment, current methods used by researchers to differentiate stem cells towards keratocytes include biochemical stimulation (culture media, growth factors, etc.), physical regulation (dome-shaped mechanical stimulation and topography), and the systematic regulation (the combination of both the physical and biochemical stimulations) (Figure 2).

5.1. Biochemical Regulation. Previously, biochemical induction of various categories of stem cells to differentiate into keratocytes has been extensively studied [1, 2, 7, 27, 29, 30, 36, 38, 41, 53, 56–58] and proven to have positive effects. Most of these studies showed significant upregulation of specific keratocyte gene expressions and some of the studies even obtained differentiated cells with keratocyte-like dendritic morphology [57] (Table 2).

As earlier mentioned, even though keratocytes can be harvested from the cornea, it is difficult to sustain their morphology and functions *in vitro* [59]. Some studies tried to achieve proliferation and the maintenance of keratocyte phenotype by culturing them on animal corneal tissue [36] or on amniotic membrane (AM) [28] which has a similar microenvironment as the cornea. Park et al. previously obtain cornea-like epithelial cells from MSCs using corneal epithelial cell-conditioned medium, which inspired them to generate a similar keratocyte-conditioned medium (KCM) in order to stimulate the differentiation of human MSCs (hMSCs) towards keratocytes [27]. KCM is a medium that is believed to contain specific biochemical factors that mimic the keratocyte microenvironment *in vivo* and thus believed to stimulate the differentiation of human stem cells into keratocytes. When hMSCs were cultured in KCM on plastic dishes, the gene expression of keratocyte markers (LUM and ALDH1A1) were increased and the expression of α-smooth muscle actin (α-SMA) was decreased [27]. This specific study is important as it is the first study to prove that it is possible to stimulate stem cell differentiation into keratocytes *in vitro* and it also emphasized the possibility of using biochemical factors for keratocyte differentiation. However, it was unknown which biochemical factor/factors in KCM played a crucial role in the keratocyte differentiation [27].

To address this question, Park et al. cultured human BMSCs with KCM supplemented with various concentrations of insulin-like growth factor binding protein 2 (IGFBP2) for 24 hours. They found increased expression of keratocyte markers (KERA and ALDH1A1) and decreased expression of myofibroblasts marker α-SMA, with the greatest effect at supplementation of 500 ng/ml of IGFBP2 [38]. In addition to this, Kafarnik et al. found that human CSSCs exposed to 10 ng/ml FGF-2 presented a keratocyte-like morphology and expression profile (increased expression of keratocyte markers, decreased expression of myofibroblastic markers and stem cell markers) [57]. Similarly, Wu et al. treated CSSCs with either 0.1 ng/ml TGF-β3, 10 ng/ml FGF-2, or a combination of both factors for 9 weeks. It was found that FGF-2 and TGF-β3 had a synergistic effect on keratocyte differentiation [53] and resulted in superior cell viability as compared to the cells that had only been added with TGF-β3 or FGF-2 and the cells exhibited a dendritic morphology similar to keratocytes. Additionally, the expression of keratocyte marker ALDH3A1 and carbohydrate sulfotransferase 6 (CHST6) was significantly increased and the expression of myofibroblast (α-SMA) was inhibited. Simultaneous stimulation of FGF-2 and TGF-β3 on human CSSCs also induced multilayered lamellae with orthogonally oriented collagen fibrils, mimicking the human corneal stromal tissue, which was not seen when FGF-2 or TGF-β3 were given separately [53]. Presently, there are mainly two typical types of keratocyte differentiation media (KDM). One is composed of advanced DMEM, ascorbate-2-phosphate (A2-P), FGF-2, and TGF-β3 [41]. The other differentiation media is mainly based on advanced DMEM, FGF-2, and A2-P [2]. This implies that the composition and concentration of growth factors in the different medium could be slightly different in different studies even though the main components of KDM are the same. The induction of keratocyte differentiation by KDM has been confirmed in various kinds of stem cells, including ASCs [1], BMSCs [36], CSSCs, [30], induced pluripotent stem cells (iPSC) [33, 60], and periodontal ligament stem cells (PDLSCs) [42]. According to those studies, KDM had a positive influence on stem cell differentiation into keratocytes which is supported by a dramatic increase of gene expressions of keratocyte markers (including KERA and LUM) and the deposition of orthogonally oriented collagens [3].

However, it is noted that keratocyte-like cells derived from differentiated stem cell are still different from the natural keratocytes in both the level of gene expression and the expression profile and the cell morphology [7]. New strategies are warranted to be developed to improve the efficiency of keratocyte differentiation.

5.2. Physical Regulation. In order to obtain superior keratocyte morphology and function from stem cells, attention has been focused not only on the biochemical stimuli but also to the physical environment of the corneal stroma. As mentioned, physical stimulations such as local topography and stress play significant roles in the regulation of keratocyte behavior *in vivo*. Therefore, physical regulation such as mechanical stimulation has been considered to be a possible way to stimulate stem cell differentiation into keratocytes. The positive influence of mechanical stimulation on stem cell differentiation has been widely studied in other

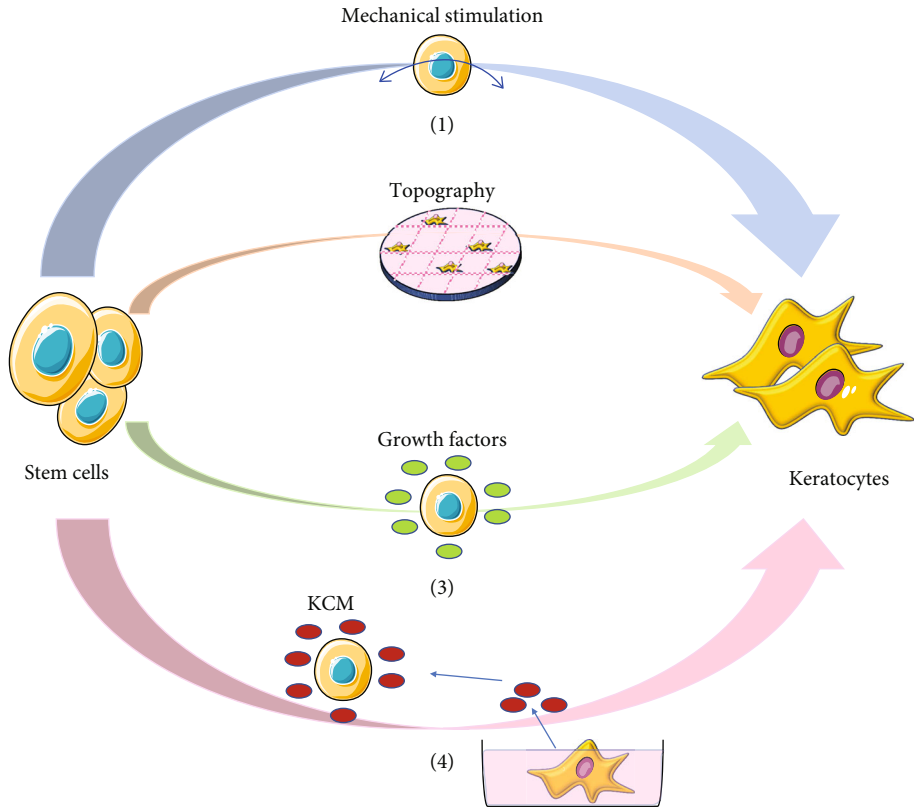

Figure 2: Current strategies for directing stem cells into keratocytes. (1) Dome-shaped mechanical stimulation; (2) topography; (3) growth factors; (4) keratocyte-conditioned medium (KCM).

Table 2: Biochemical stimulation induces keratocyte differentiation.

Stem cells	Treatments	Effects
hMSCs	KCM	Spindle shaped; upregulated gene expression of keratocyte markers (KERA and ALDH1A1); downregulated expression of fibrotic marker (α-SMA) [27]
	KCM+AM	Dendritic or stellate morphology; upregulated gene expression of keratocyte markers (KERA and ALDH1A1); downregulated expression of fibrotic marker (α-SMA) [27]
	KCM+IGFBP2	Upregulated gene expression of keratocyte markers (KERA, LUM, and ALDH1A1) and downregulated expression of fibrotic marker (α-SMA) [38]
hCSSCs	FGF-2	Keratocyte-like morphology; barely no expression of myofibroblastic marker (α-SMA); increased protein and gene expression of keratocytes (KERA, LUM, ALDH3A1); decreased protein and gene expression of stem cells (Paired Box Gene 6 (Pax6), N-cadherin) [57]
	TGF-β3	Upregulated gene expression of keratocyte markers (KERA, beta-1,3-N-acetylglucosaminyltransferase 7 (B3GnT7), CHST6) [53]
	FGF-2+TGF-β3	Upregulated gene expression of keratocyte markers (KERA, B3GnT7, CHST6) [53]
ASCs	KDM[1]+RA	Upregulated gene expression of keratocyte markers (KERA, ALDH3A1, LUM, decorin); higher acid sulfated glycosaminoglycans' secretion; decreased expression of fibrotic marker (α-SMA) [2]
LBSCs	KDM[2]	Decreased expression of the stem cell genes (adenosine triphosphate-binding cassette G2 (ABCG2) and Nestin); increased gene expression of keratocyte markers (ALDH3A1, aquaporin1 (AQP1), KERA, and prostaglandin D2 synthase); secretion of ECM [30]
hPDLSCs	KDM[2]	Upregulated gene expression of keratocyte markers (LUM, KERA, ALDH3A1, ALDH1A1, COL I, COL V, COL III, COL VI) [7]

KCM: keratocyte-conditioned medium; KDM[1]: keratocyte differentiation media which consists of advanced DMEM, ascorbate-2-phosphate (A2-P), and 10 ng/ml fibroblast growth factor-2 (FGF-2); KDM[2]: keratocyte differentiation media which consists of advanced DMEM, A2-P, 10 ng/ml FGF-2, and 0.1 ng/ml transforming growth factor-β3 (TGF-β3); AM: amniotic membrane; RA: retinoic acid; IGFBP2: insulin-like growth factor binding protein 2.

tissues and cell types, including chondrogenesis [61] and differentiation into tenocytes [62]. Unlike other tissues, corneal stroma is under dome-shaped strain. Therefore, Chen et al. applied a dome-shaped static mechanical stimulation, mimicking the *in vivo* cornea, and discovered that it was able to upregulate the expression of ALDH3A1, CD34, LUM, COL I, and COL V in PDLSCs [3]. The dome-shaped mechanical stimulation also had a synergistic effect in combination with KDM. The results of this study suggest that physical strategies are a promising method in the regulation of keratocyte differentiation.

Additionally, topographical cues in the microenvironment are essential in the regulation of cell behaviors during both the physiological and pathological conditions. The arrangement and the length of the ECM components are able to modulate a broad range of cell behaviors like adhesion, morphology, and differentiation [55]. In the corneal stroma, the highly organized environment composed of a unique arrangement of collagen lamellar has been proven to be significant in obtaining keratocyte morphology and offers a topographical cue to the differentiation of keratocytes [63, 64]. Teixeira et al. cultured human keratocytes on silicone substrate containing grooves and ridges, ranging from 70 to 2000 nm which strongly align the keratocytes in the direction of the anisotropic patterns. Generation of focal adhesions and stress fibers by keratocytes was reduced on 70 nm-wide ridges when compared to micron-size patterns or smooth substrates. This observation indicated that the keratocytes are sensitive to the anisotropic topographic stimuli and that suitable substrate topographies are able to affect the behavior of keratocytes such as the keratocyte-myofibroblast transdifferentiation [63]. Moreover, hCSSCs were cultured on both aligned and random polyurea fibers in KDM to explore the role of topography in inducing an ECM secretion profile similar to that of the native corneal stroma. Wu et al. found that hCSSCs differentiated into keratocytes seeded on aligned substrates secreted more ECM, similar to the native corneal stroma [64]. Thus, the physical environment is essential for the maintenance of both the function and the behavior of keratocytes and offers a clue for the differentiation of stem cells towards functional keratocytes.

5.3. Systematic Regulation. Since neither biochemical regulation nor physical regulation alone is able to achieve the desired effect, researchers tend to combine the two approaches to achieve more sufficient differentiation result (Table 3). Yam et al. cultured PDLSCs in a 3D pellet model (cells were suspended in a spherical shape) and subsequently induced differentiation with KDM [6]. The pellet model is considered to increase cell-to-cell interactions (mainly cadherin-containing and connexin-containing junctions) [31] and was accepted as an appropriate model since it promotes production of total collagen and expression of KERA, the major proteoglycan in corneal stroma [65]. In Yam et al.'s study, they used the pellet model with KDM and more specifically CSK (corneal stroma keratocyte) induction media, which is mainly based on FGF-2, TGF-β3, and DMEM/F12. They found that the stimulation upregulated the expression of keratocyte-specific markers (ALDH3A1, KERA, LUM, CHST6, B3GNT7, and Col8A2) and downregulated the expression of genes related to fibrosis and other lineages [6]. Some researchers also induced totipotent stem cells (human iPSCs [33] and ESCs [34]) into keratocytes by using the method of combining both the physical and the biochemical strategies. As both iPSCs and ESCs usually are difficult to differentiate into specialized mature cells, it is performed in a two-step procedure by first inducing them into neural crest lineage before they are differentiated into keratocytes. Naylor et al. conducted the two-step procedure to gain keratocyte-like cells from human iPSCs. They first cocultured the human iPSCs with bone marrow stroma cell line such as PA6 or Ms5 supplemented with FGF-2 in order to obtain neural crest lineage cells (NCCs) [33]. Subsequently, they used the NCCs either in the pellet model with KDM, which is similar to the real corneal microenvironment, or in seeding the cells on the sclera of a corneal rim slice. The gene expression of keratocyte markers from NCCs using the pellet model was 10-folds higher than the control group. The NCCs cultured on the sclera of corneal rims had even higher gene expression of keratocyte marker and reached an expression level similar to what is found in human corneal keratocytes [33]. Chan et al. cocultured human ESCs with PA6 to gain NCCs before culturing the NCCs in the pellet model with KDM, which resulted in upregulated gene expression of keratocyte-specific markers (including KERA, AQP1, ALDH3A1, CHST6, B3GNT7, and PTGDS), as well as the cells exhibiting a keratocyte-like dendritic morphology suggesting that the cells deriving from human ESCs were more sufficient as compared to iPSCs [32]. Kong et al. constructed a highly aligned 3D microfibrous scaffold similar to the physical structure of the human cornea. CSSCs were cultured on the scaffold with chemical factors including serum, insulin, FGF-2, and ascorbic acid for 2 weeks and found both an upregulation of the gene expression of keratocyte specific markers and a downregulation of fibrotic genes [66].

6. Perspectives

Stem cell differentiation into keratocytes has lately experienced great progression regarding the efficiency, mainly by applying either biochemical factors, physical regulation, or the combination of both. However, the keratocyte-like cells derived from differentiated stem cells are still not ideal for corneal tissue engineering as the gene expression levels of keratocyte-specific markers and proteoglycans are usually much lower than those of native keratocytes [6]. Additionally, current research lack a more refined system for evaluating whether stem cells have fully differentiated into keratocytes, which needs to be improved in the future. To generate a more efficient differentiation strategy, we propose the following possible directions in the research field:

First, novel biochemical factors need to be unravelled. Even though the recent keratocyte differentiation medium, mainly composed of FGF-2 and TGF-β3, is able to induce stem cell differentiation into keratocyte-like cells, it still does not reach the phenotype level as seen in the native corneal

Table 3: Systematic regulation induces keratocyte differentiation.

Stem cells	Systematic regulation	Findings
hESCs	Stem cells cocultured with mouse PA6 fibroblasts in serum-free medium containing ascorbate in order to generate NCCs. Subsequently, NCCs were cultured in the pellet model supplemented with KDM	Upregulated gene expression of keratocyte markers (AQP1, B3GNT7, PTDGS, and ALDH3A1); increased secretion of corneal-specific proteoglycan [32]
hiPSCs	Stem cells cocultured with bone marrow stroma cell line such as PA6 or MS5, supplemented with FGF-2 to generate NCCs. Subsequently, NCCs were cultured in the pellet model supplemented with KDM	Upregulated gene and protein expression of keratocyte markers (ALDH3A1, KERA, PTDGS, AQP1, CHST6) [33]
	Stem cells were seeded onto the sclera of corneal rim slice (specific niche including both the physical and biochemical regulations)	Keratocyte-like morphology; upregulated gene expression of keratocyte markers (ALDH3A1, KERA, PTDGS, AQP1, CHST6) [33]
hPDLSCs	Stem cells cultured in the pellet model supplemented with CSK induction media	Keratocyte-like morphology; upregulated gene expression of keratocyte markers (CD34, ALDH3A1, KERA, LUM, CHST6, B3GNT7, Collagen Type VIII Alpha 2 Chain (Col8A2)) [6]
	Stem cells cultured in the pellet model on human amnion stroma (specific niche including both the physical and biochemical regulations)	Keratocyte-like morphology; suppression of fibroblast genes (α-SMA); upregulated gene expression of keratocyte markers (CD34, ALDH3A1, KERA, LUM, CHST6, B3GNT7, Col8A2) [6]
	Stem cells cultured on porcine corneal stroma (specific niche including both the physical and biochemical regulations)	Presence of keratocyte gene expression (CD34, ALDH3A1, KERA, LUM, CHST6, B3GNT7, Col8A2); negligible fibroblast gene expression (α-SMA) [6]
ASCs	Stem cells cultured on fibrin gel supplemented with KDM	Presence of the stroma-specific ECM molecules; keratocyte-like cells; presence of less consistent expression of both KERA and keratan sulfate at protein and mRNA level [1]
	Stem cells cultured in the pellet model supplemented with KDM	Presence of the stroma-specific ECM molecules; keratocyte-like cells; presence of more consistent expression of both KERA and keratan sulfate at protein and mRNA level [1]
	Stem cells cultured on fibrin gel supplemented with KDM	Lower level of KERA mRNA compared with that cultured in pellet; presence of the stroma-specific ECM molecules [1]
hCCSCs	Stem cells were cultured on highly aligned 3D gel MA hydrogel scaffold with the supplementation of chemical factors (serum, insulin, FGF-2, and ascorbic acid)	Upregulated expression of keratocytes' genes (KERA, AQP1, and ALDH3A1); Downregulated expression of fibroblastic genes (α-SMA) [66]
hMSCs	Stem cells were transplanted into mice's cornea	Upregulated expression of keratocytes' mRNA (KERA and LUM) [36]

NCCs: neural crest cells; KDM: keratocyte differentiation media which consist of advanced DMEM, ascorbate-2-phosphate (A2-P), and 10 ng/ml fibroblast growth factor-2 (FGF-2); CSK: corneal stroma keratocyte induction media which consist of DMEM/F12, insulin-selenate-transferrin, ascorbate-2-phosphate (A2-P), 20 ng/ml fibroblast growth factor-2 (FGF-2), and 0.1 ng/ml transforming growth factor-β3 (TGF-β3).

keratocytes [53]. Additionally, it is problematic to prepare KDM as it usually consists of several components, and the stem cells need at least 14 days to differentiate into keratocyte-like cells. Thus, it is of great interest to find novel and more effective biochemical factors to improve the differentiation efficacy. By comparing the expression profile of corneal tissue with other tissues to unravel what is specifically expressed in corneal tissue, important for the homeostasis of corneal tissue, might be a feasible method to discover and isolate key biochemical factors. It may also be useful to thoroughly explore the embryonic development of corneal stroma to map key factors in the process. Furthermore, cell fate is known to be regulated at multiple levels, which makes joint analysis of multiomics (epigenetic, transcriptomics, proteomics, etc.) a promising approach to better discover biochemical factors to stimulate differentiation of stem cells into keratocytes.

Secondly, when it comes to physical regulation, there are more aspects that can be considered to mimic the microenvironment of the native corneal keratocytes, such as the stiffness. A previous study has shown that stem cells will undergo lineage-specific differentiation when cultured on substrate that has similar stiffness as the native microenvironment [67]. For example, MSCs will be able to undergo superior osteogenesis capacity when cultured on 20 kPa substrate as compared to 2 kPa substrate [52]. When it comes to the corneal stroma, the physiological stiffness of the human cornea is around 24-39 kPa [68] which is much softer than the commonly used culture plate that has around 10^6 kPa. Chen et al. found that the substrate stiffness of 25 kPa, which is similar to the natural cornea tissue, represented a positive effect on maintaining phenotype of cultured keratocytes [69]. Hence, using specific methods to adapt the stiffness of cell-culturing

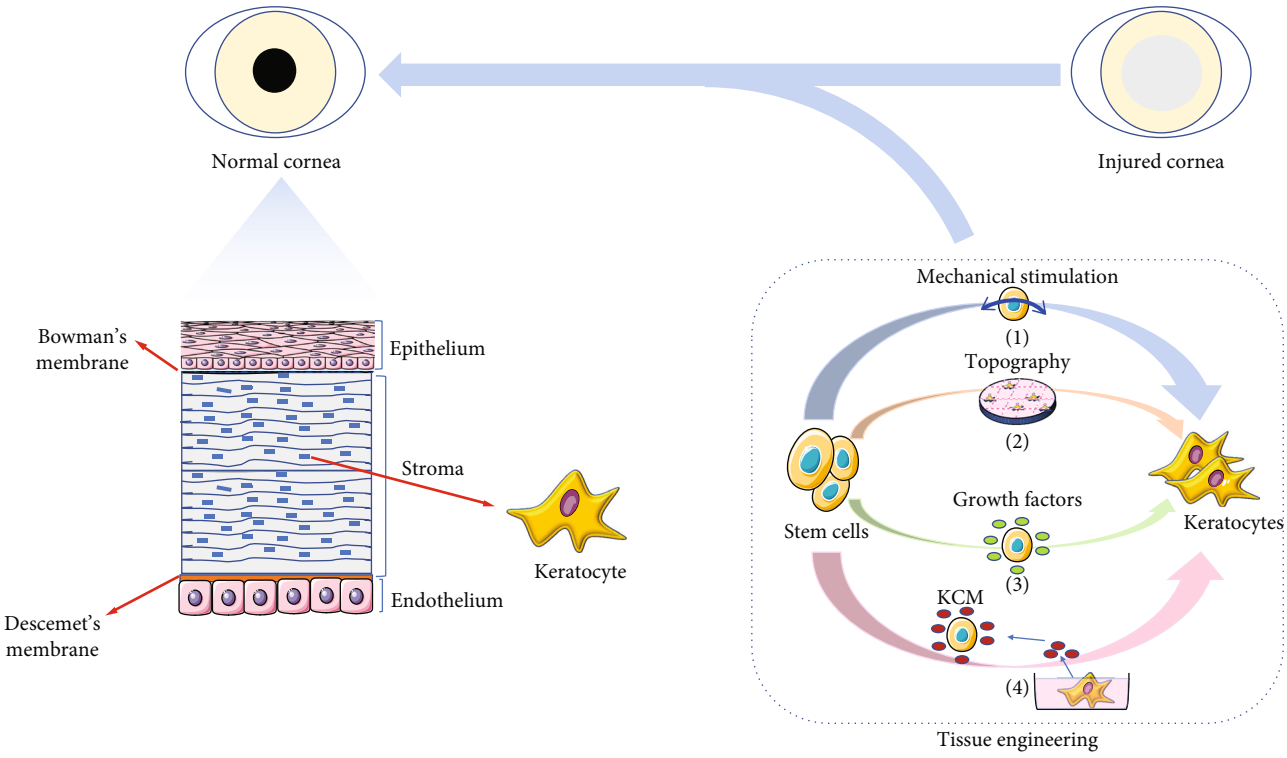

Figure 3: Schematic diagram summarizing the whole scope of the article. The normal cornea consists of five layers—the epithelium layer, Bowman's membrane, the stroma layer, Descemet's membrane, and the endothelium layer. We summarized the tissue engineering strategies that can make it possible to restore the damaged cornea to a normal cornea.

substrate might be a promising method to induce a more sufficient differentiation of stem cells into keratocytes. To further speculate, combining the regulation of other physical properties of scaffold material (such as the highly organized alignment of material) with the modification of stiffness may lead to synergetic effect on the differentiation of stem cells into keratocytes.

7. Conclusions

This review evaluates the roles of biochemical factors, physical regulation, and a combination of both in stem cell differentiation into keratocyte (Figure 3). For the biochemical approach, combinations of cytokines are used to achieve a more sufficient differentiation of stem cells into keratocytes. Regarding the physical regulation, an attempt to mimic the stress and alignment of collagen fibers of the native corneal microenvironment is achieved to improve the differentiation of stem cells into keratocytes. However, current methods to induce stem cells to differentiate into keratocytes still have their limitations as the level of keratocyte-specific genes and expression of proteoglycans is lower than that of native keratocytes. Suggested future progression is to perform attempts of finding novel effective biochemical factors either by performing in-depth analysis of factors in corneal tissue as compared to other tissues to unravel what is specifically expressed in corneal tissue or by doing a meticulous exploration of factors involved in the embryotic development of corneal stroma. A valuable aspect would be to combine the tissue-specific physical regulations in order to accomplish an experimental microenvironment that mimics the *in vivo* environment in corneal keratocytes by regulating the stiffness, topography, and other physical properties of the substrate in the experimental microenvironment.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Aini Zhang and Wei Zhang contributed equally to this work.

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Review Article

Ginsenoside Rg1 as a Potential Regulator of Hematopoietic Stem/Progenitor Cells

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Ginsenoside Rg1 (Rg1), a purified, active component of the root or stem of ginseng, exerts positive effects on mesenchymal stem cells (MSCs). Many recent studies have found that hematopoietic stem cells (HSCs), which can develop into hematopoietic progenitor cells (HPCs) and mature blood cells, are another class of heterogeneous adult stem cells that can be regulated by Rg1. Rg1 can affect HSC proliferation and migration, regulate HSC/HPC differentiation, and alleviate HSC aging, and these findings potentially provide new strategies to improve the HSC homing rate in HSC transplantation and for the treatment of graft-versus-host disease (GVHD) or other HSC/HPC dysplasia-induced diseases. In this review, we used bioinformatics methods, molecular docking verification, and a literature review to systematically explore the possible molecular pharmacological activities of Rg1 through which it regulates HSCs/HPCs.

1. Introduction

Ginsenosides are the active components of ginseng and comprise a group of sterol compounds. According to differences in their glycosidic structure, ginsenosides are divided into two subtypes: the dammarane type and the oleanane type [1, 2]. Ginsenoside Rg1 (Rg1, molecular formula: $C_{42}H_{72}O_{14}$, Figure 1(a), image from PubChem), a member of the ginsentriol subtype of dammarane ginsenosides, is an important monomeric ginsenoside and the most abundant component of Chinese/Korean ginseng. Rg1 not only acts on the nervous, cardiovascular, blood, and immune systems but also exhibits a variety of positive pharmacological activities, such as its neuroprotective activity [3] and its abilities to treat myocardial ischemia [4] and repair hematopoietic immune disorders [5]. To date, several clinical trials on the use of drugs containing Rg1 for the treatment of vascular dementia, hyperlipidemia, hypertension, Sjögren's syndrome, rheumatic diseases, and ischemic stroke have been registered on clinicaltrials.gov [6, 7].

Hematopoietic stem cells (HSCs) are adult stem cells that can self-renew, differentiate into blood cell lineages, and exert long-term effects on maintaining and producing all mature blood cell lineages during the life cycle of an organism [8]. Under a stable metabolism status, most HSCs are in a static state (quiescent HSCs), whereas hematopoietic progenitor cells (HPCs) actively proliferate and maintain the daily hematopoietic function. When the body is stimulated, such as during life-threatening blood loss, infection, and inflammation, HSCs can be activated in the bone marrow to proliferate and participate in blood formation [9, 10]. HSCs mainly function in the specific bone marrow microenvironment (HSC niche), which provides the signals needed to protect HSCs and maintain HSC differentiation [11]. Although the surface phenotypes of human and murine HSCs differ, these cell types possess the basic functions of HSCs. CD34 is a marker of human HSCs, and clinical transplantation studies using enriched $CD34^+$ bone marrow cells have indicated the presence of HSCs with the ability to reconstitute bone marrow within this fraction [12, 13]. For differentiation and functional

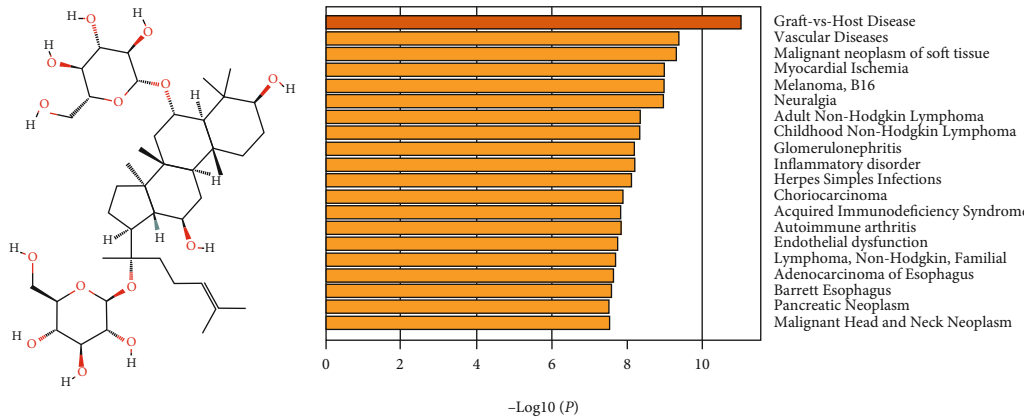


FIGURE 1: Molecular structure of Rg1 and the diseases that may be targeted by Rg1. (a) Molecular structure of ginsenoside Rg1. (b) GVHD is the top putative disease targeted by Rg1, as shown by an analysis using the Metascape platform.

research involving murine HSCs, a variety of markers are commonly used to identify or isolate HSCs. In general, murine HSCs do not express lineage-specific markers (Lin^-) and are positive for c-kit ($CD117^+$ or $c\text{-}Kit^+$) and stem cell antigen-1 ($Sca\text{-}1^+$). More recently, the HSC population was further clarified to include the markers CD150 and CD48, and as a result, this population was defined by the marker profile $Lin^-c\text{-}Kit^+Sca\text{-}1^+CD48^-CD150^+$ [14–16].

In a previous study, we described the active regulatory effects of Rg1 on the proliferation, differentiation, senescence, and apoptosis of mesenchymal stem cells (MSCs) [17]. Here, HSCs, a heterogeneous adult stem cell population that completely differs from MSCs, were analyzed and systematically reviewed. We used bioinformatics methods to analyze the potential molecular pharmacological role of Rg1 in HSC/HPC regulation and reviewed the literature to summarize the mechanisms through which Rg1 activates HSC proliferation and differentiation and its antiaging effects in HSCs/HPCs.

2. Prediction of Potential Rg1 Targets in HSCs/HPCs Based on a Bioinformatics Analysis

The molecular structure of Rg1 from PubChem was analyzed to identify putative targets of Rg1, and the TargetNet and SwissTargetPrediction platforms were also used to predict putative targets of the ginsenoside Rg1 [18, 19]. A comprehensive search identified 723 putative targets (623 from TargetNet and 100 from SwissTargetPrediction) of Rg1 (Supplementary Materials 1 and 2). Furthermore, the top 20 targets (10 from TargetNet and 10 from SwissTargetPrediction) with relatively high probability among the putative targets were pooled and used to predict associated diseases that may be regulated by Rg1 (analyzed by DisGeNET using the Metascape platform [20]). The results showed that graft-versus-host disease (GVHD) is the top putative targeted disease that may be regulated by Rg1 (Figure 1(b)). This finding suggests that Rg1 could be used as a potential monomeric drug to reduce GVHD and that Rg1 could further improve the success rate of HSC transplantation.

To perform an interactive bioinformatics analysis of the relationships between HSC proliferation and migration and Rg1, we analyzed the molecular functions of Rg1 through a metaenrichment of pathway. Genes that may be related to HSC proliferation or migration were analyzed using the Comparative Toxicogenomics Database, and we identified 49 genes associated with HSC proliferation and 23 genes (after removing repetition) associated with HSC migration (Supplementary Materials 3 and 4). A metaenrichment analysis of Rg1 and HSC targets was performed using the Metascape platform, and the results showed that G protein-coupled receptor binding was enriched in the effects of Rg1 on HSC proliferation, whereas integrin binding and protein homodimerization activity were enriched in the effects of Rg1 on HSC migration (Figure 2).

In addition, we used bioinformatics methods to analyze the potential molecular biological functions of Rg1 during the process of HSC or HPC differentiation. Putative targets of HSC or HPC differentiation were obtained using the Comparative Toxicogenomics Database. A total of 183 and 359 genes (after removing repetition) related to HSC and HPC differentiation, respectively, were identified (Supplementary Materials 5 and 6). The results of the metaenrichment analysis of pathways using the Metascape platform showed that transcription factor binding and endopeptidase activity are enriched molecular functions in HSC differentiation that may be regulated by Rg1. Moreover, protein domain-specific binding was found to be an additional molecular function that may be regulated by Rg1 during HPC differentiation (Figure 3).

Interestingly, the use of JVenn to visualize the specific targets through which Rg1 regulates HSCs [21] revealed that angiotensin-converting enzyme (ACE, GeneID: 1636) was the only overlapping gene through which Rg1 regulates HSC proliferation and HSC/HPC differentiation (Figure 4). We then used molecular docking to verify the interaction between Rg1 and ACE. Briefly, the crystal structures of putative targets were obtained from the Protein Data Bank, and AutoDock Tools 1.5.6-Vina software was used for the analysis of binding ability and sites. Additionally, PyMOL was utilized to visualize

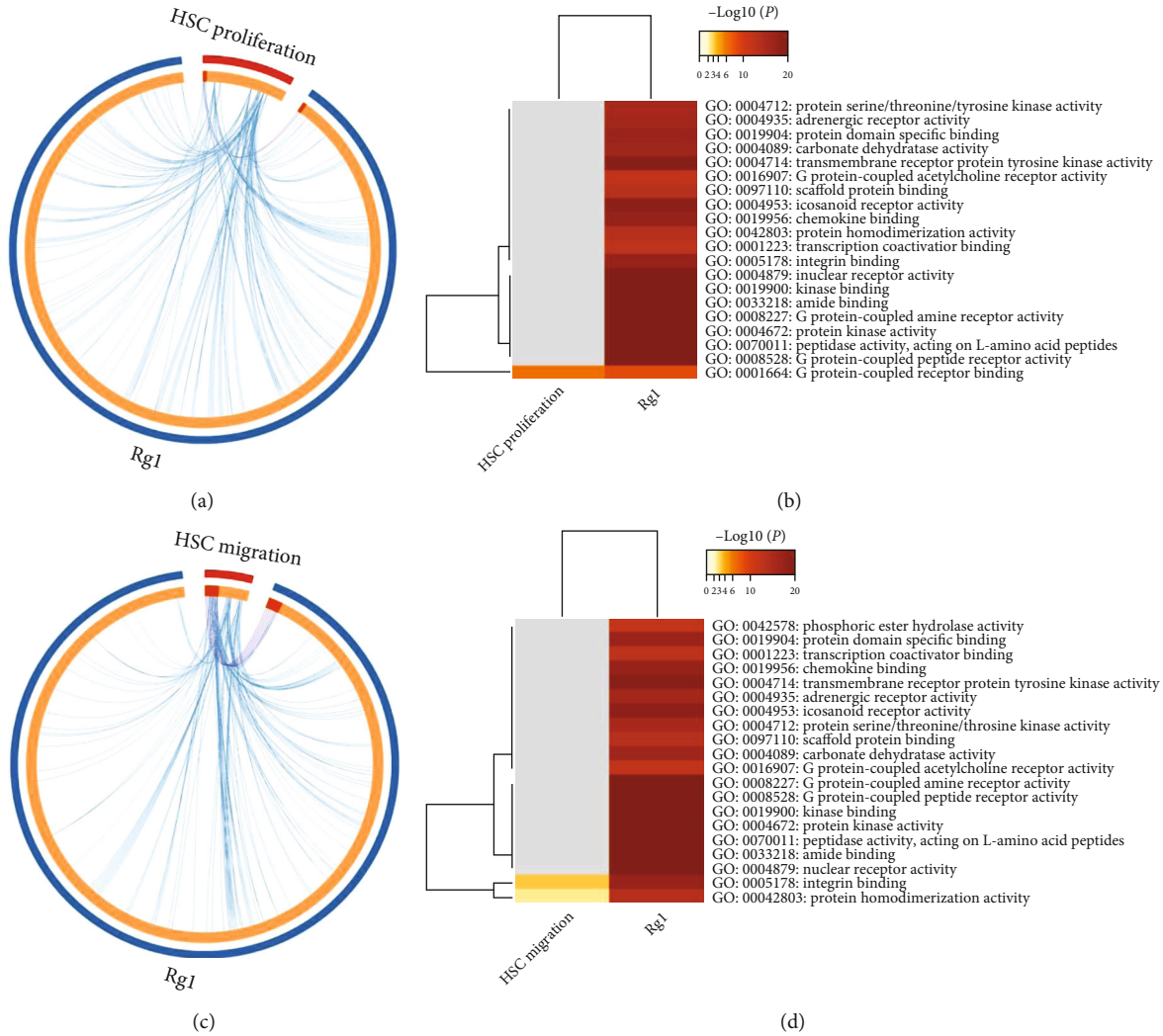


Figure 2: Metaenrichment analysis of the molecular effects of Rg1 on HSC proliferation or migration. (a) Ring summary shows overlapping genes related to Rg1 and HSC proliferation at the gene level (the purple line shows the overlapping genes; the blue line shows a functional correlation between genes). (b) Heatmap of terms enriched in the list of genes targeted by Rg1 to regulate HSCs. The terms are colored based on the p value. (c) Ring summary of overlapping genes through which Rg1 regulates HSC migration at the genetic level. (d) Heatmap of terms enriched in the genes targeted by Rg1 to regulate HSC migration. The terms are colored based on the p value.

the interaction between Rg1 and the ACE peptide chain. The results showed that several hydrogen bonds may form between Rg1 and ACE. Specifically, the use of the minimum binding energy (affinity: -13.0 kcal/mol) in the docking analysis revealed that hydrogen bond formed between Rg1 and threonine in chain A of ACE (Figure 4).

Furthermore, the signal pathways and molecular mechanism in the bioinformatics results provide some novel research directions and may be worth further exploration in in vitro/in vivo experiments. All the databases used in the study are listed in Supplementary Material 7.

3. A Review of the Literature Reveals That Rg1 Regulates HSC Proliferation, Differentiation, and Migration

HSCs differentiate into myeloid progenitor cells and prolymphoid progenitor cells in the bone marrow to drive bone marrow hematopoiesis [11, 22]. A previous study found that Rg1 can regulate calcium-sensing receptor (CaSR) to increase the number of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ HSCs and lymphoid $CD3^{+}$ cells in the bone marrow and peripheral blood of CY-induced myelosuppressed mice and thereby restores bone marrow function [23]. Interestingly, an analysis of the 9 overlapping target genes through which Rg1 regulates HSC differentiation using the Metascape platform revealed that the calcium signaling pathway was the key KEGG pathway through which Rg1 regulates HSC differentiation (Figure 5(a)). This consistent finding confirms that the CaSR-mediated calcium signaling pathway may be a crucial target through which Rg1 regulates HSC differentiation.

In addition, some studies have shown that Rg1 improves the hematopoietic activity of the bone marrow through extramedullary hematopoiesis. Cyclophosphamide (CY) can cause bone marrow cytotoxicity, leading to bone marrow suppression and triggering extramedullary

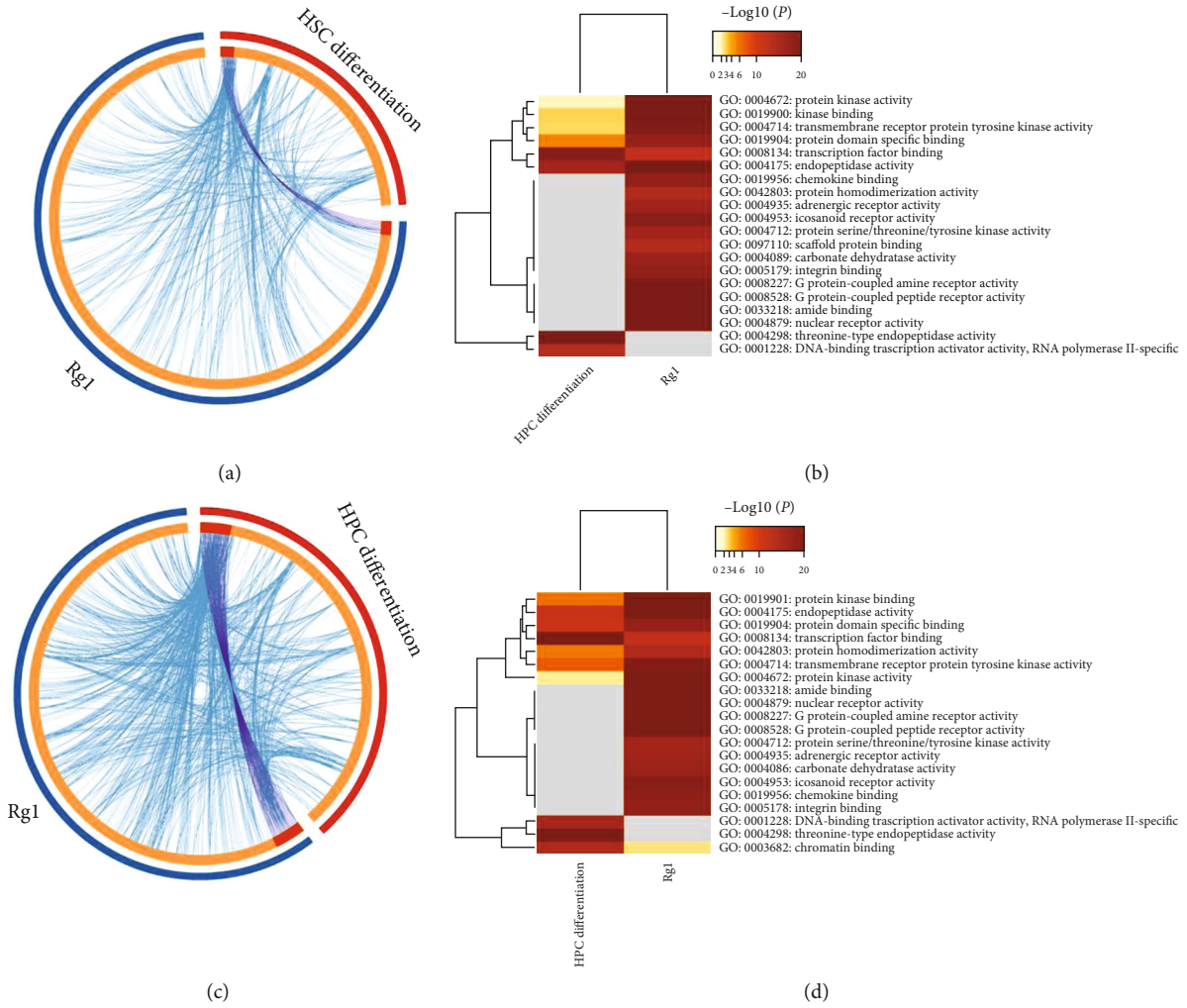
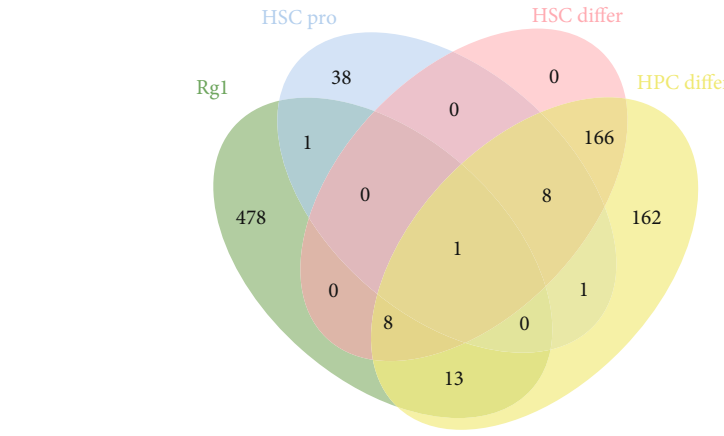


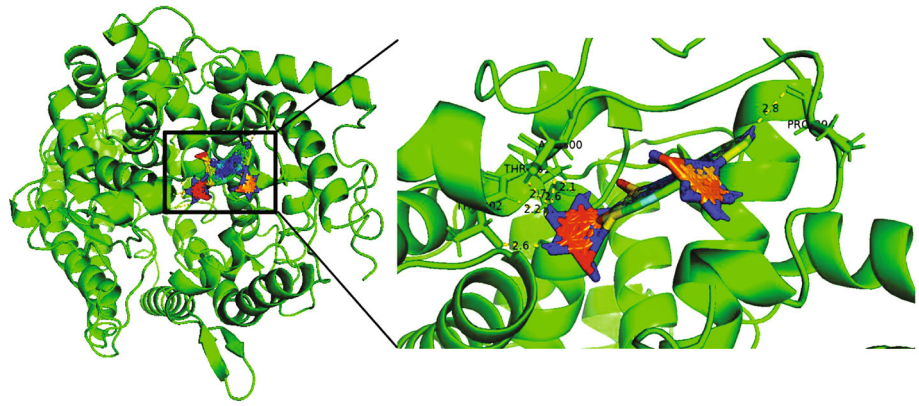
Figure 3: Metaenrichment analysis of the molecular effects of Rg1 on HSC/HPC differentiation. (a) Ring summary of overlapping genes through which Rg1 regulates HSC differentiation at the genetic level (the purple line shows overlapping genes; the blue line shows a functional correlation between genes). (b) Heatmap of terms enriched in the genes targeted by Rg1 to regulate differentiation. The terms are colored based on the p value. (c) Ring summary of overlapping genes through which Rg1 regulates HPC differentiation at the genetic level. (d) Heatmap of terms enriched in the genes targeted by Rg1 to regulate HPCs differentiation. The terms are colored based on the p value.

hematopoiesis [24, 25]. Extramedullary hematopoiesis is characterized by the presence of pluripotent HPCs, including erythroid lineage cells, myeloid lineage cells, and megakaryocytes, in the spleen and liver [26]. Liu et al. [27] found that Rg1 treatment could effectively reduce the weight of the spleen of CY-stimulated mice and reduce the absolute number of c-Kit$^+$ HSCs in the spleen and that these effects are not caused by apoptosis, which suggests that Rg1 alleviates CY-induced extramedullary hematopoiesis in the spleen. Further research shows that Rg1 could upregulate the proliferative activity of c-Kit$^+$ HSCs in the spleen but not in the bone marrow of CY-stimulated mice. Moreover, Rg1 increases the number of c-Kit$^+$/CD45$^+$ HSCs in the peripheral circulatory system. Most importantly, the effect of Rg1 on HSCs in the bone marrow and peripheral blood is not observed in splenectomy- and CY-induced mice. These results systematically indicate that Rg1 improves CY-induced myelosuppression by activating HSC proliferation in the spleen, particularly by allowing the homing of HSCs from the spleen through the circulatory system to the bone marrow [27]. In addition, the selective regulation of HSCs in the spleen but not in the bone marrow also suggested that the "spleen-bone marrow" axis homing of HSCs plays a main/crucial role in Rg1 relieving extramedullary hematopoiesis and myelosuppression. Moreover, quiescent HSCs in bone marrow HSC "niche" can maintain hematopoietic homeostasis [28]. In the above study, after the new "niche" formed, the homing HSCs derived from the spleen may also serve as quiescent HSCs and further benefit bone marrow hematopoietic homeostasis.

Interestingly, whether Rg1 directly activates and promotes the proliferation of quiescent HSCs in the bone marrow niche was an open question. First, CD34$^+$ cells account for only 1.5% of human bone marrow mononuclear cells, and murine Lin$^-$Sca-1$^+$c-Kit$^+$ HSCs account for less than 1% of bone marrow cells. The treatment of mice with CY



(a)



(b)

Figure 4: Common proteins among putative proteins targeted by Rg1 and proteins related to HSC proliferation, HSC differentiation, and HPC differentiation. (a) Venn diagram showing that one protein (ACE) was found in all four lists. (b) Three-dimensional schematic representation showing the molecular docking model, active sites, and binding distances for Rg1 and ACE after the application of ray tracing.

results in the appearance of a large amount of vacuole-like degradation in the bone marrow cavity and a sharp decrease in the number of bone marrow cells (including MSCs) [23, 29]. In this context, very few HSCs remain in the bone marrow, and the effect of Rg1 on enhancing the mobilization of extramedullary hematopoiesis far exceeds the effect of Rg1 on the mobilization of HSCs in the bone marrow. Therefore, the direct effect of Rg1 on quiescent HSCs in the bone marrow may be difficult to observe. Second, the researchers continuously administered Rg1 (15 mg/kg/day) to CY-induced myelosuppressed mice (splenectomy) for 7 days [27], and the results showed that Rg1 could not effectively increase the percentage of bone marrow $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ HSCs (no significant difference), but an increasing trend was observed. Third, Rg1 could also regulate MSCs to protect HSCs from D-galactose- (D-gal-) induced damage [30], and the continuous administration of Rg1 for 7 days could not completely restore the histological morphology of the murine bone marrow (vacuolar pathological structures remained in the bone marrow cavity) [23]. Thus, we infer that the regulatory effect of MSCs on the hematopoietic microenvironment could be delayed. Therefore, further investigation of whether Rg1 can activate HSCs in the bone marrow by prolonging the duration of Rg1 administration to explore the recovery of bone marrow HSCs in mice with splenectomy is warranted (Figure 5(b)).

In addition, stromal-derived factor-1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR4) is an important signaling molecule in HSC homing to the bone marrow and bone marrow implantation [31]. Rg1 can regulate the SDF-1α/CXCR4 axis and plays a regulatory role in the vascular intima [32]. These findings also suggest that Rg1 promotes HSC homing from the spleen to the bone marrow cavity and exerts hematopoietic effects in the bone marrow.

4. Mechanisms Involved in the Attenuation of HSC Aging by Rg1

Traditionally, aging HSCs gradually lose the potential for self-renewal and differentiation, and the likelihood of abnormal metabolic cellular functions greatly increases [33]. An increasing number of studies have shown that inflammation and chemical or physical factors also cause DNA damage, which can lead to HSC aging [10, 34, 35]. Excessive D-gal results in the production of aldohexose and hydrogen peroxide via galactose oxidase and promotes the generation of

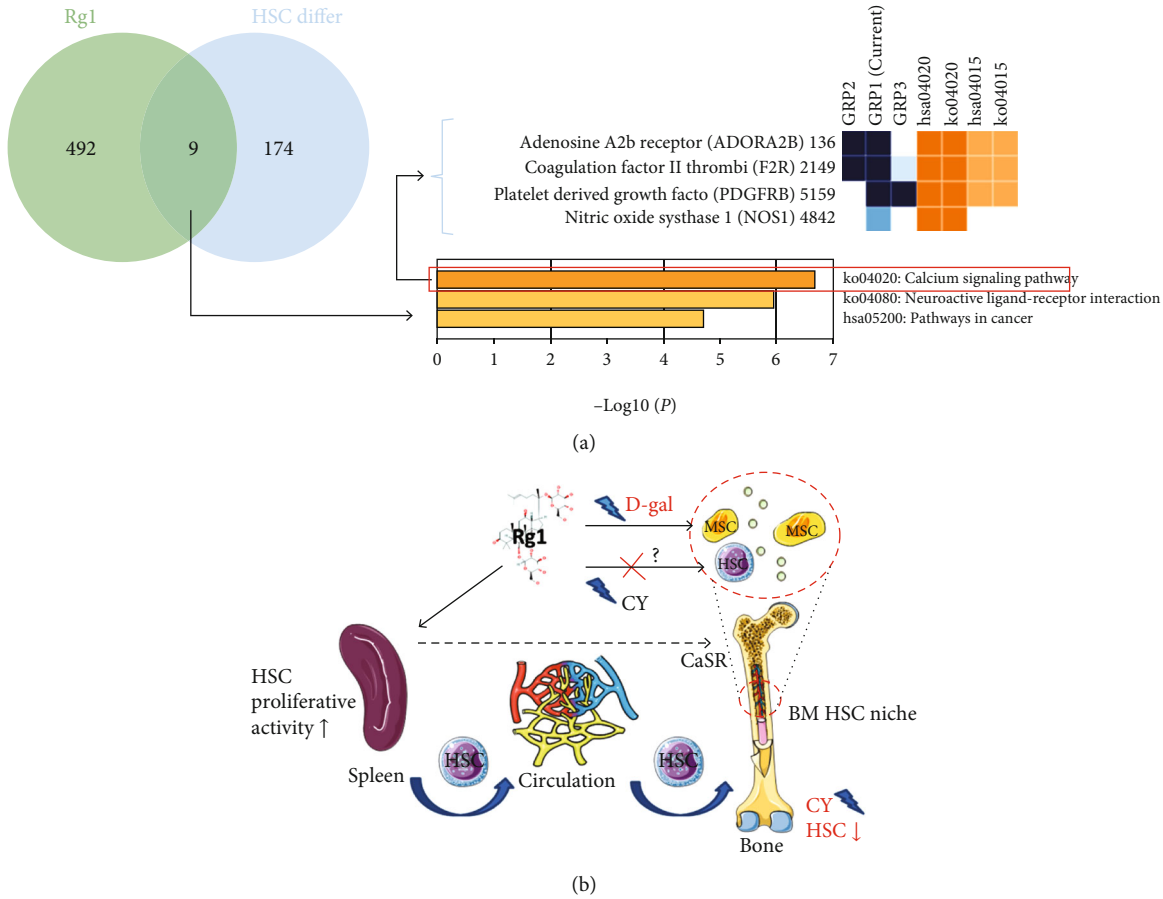


Figure 5: Relevant bioinformatics data and mechanistic summary of the mechanism through which Rg1 regulates the proliferation, differentiation, and migration of HSCs. (a) Venn diagram and KEGG pathway enrichment analyses revealed that the calcium signaling pathway is the key pathway through which Rg1 regulates HSC differentiation. (b) Rg1 promotes the homing of HSCs from the spleen to the bone marrow through the peripheral circulatory system in mice with myelosuppression caused by cyclophosphamide. CY: cyclophosphamide; HSC: hematopoietic stem cell; D-gal: D-galactose; CaSR: calcium-sensing receptor; MSC: mesenchymal stem cell.

oxygen-derived free radicals and superoxide anions, which results in impairment of the functions of macromolecules and cells [36, 37]. Rg1 inhibits oxidative stress and reduces DNA damage, which results in enhancement of the antiaging ability of Sca-1^{+}HSCs/HPCs in a murine model of D-gal-induced aging, and the effect is related to inhibition of excessive activation of the Wnt/β-catenin signaling pathway. The classic Wnt/β-catenin pathway is essential for the regulation of stem cell pluripotency and the determination of cell fate [38, 39]. When D-gal activates the Wnt/β-catenin pathway, the Wnt ligand (a secreted glycoprotein that binds to Frizzled receptors) forms a large cell surface complex with low-density lipoprotein receptor-related protein (LRP) 5/6. A previous study found that Rg1 can inhibit D-gal-induced overactivation of the Wnt/β-catenin signaling pathway. Rg1 can reduce β-catenin expression and glycogen synthase kinase 3 beta (GSK3β) phosphorylation in the cytoplasm and can further reduce the protein expression of β-catenin in the nucleus via Ras-related C3 botulinum toxin substrate 1 (Rac1) and other factors; in addition, the binding of β-catenin to the transcription factor TCF-4 in the nucleus is reduced and ultimately inhibits c-Myc gene expression, which results in the reduction of β-galactosidase expression [40] (Figure 6).

A previous study also showed that Rg1 could mediate the p53-p21-Rb signaling pathway to improve routine blood index abnormalities caused by lead acetate and alleviate lead acetate-induced HSC aging and aging-related inflammatory responses. Lead acetate can cause DNA damage in HSCs and induce cells to produce γ-H2AX. Rg1 can reduce the DNA damage-induced increases in p53 transcription and translation but does not affect the activity of P16, which results in the amelioration of lead acetate-induced HSC damage [41]. Studies also found that Rg1 can attenuate ROS production to improve HSC function in various settings [40, 42–44]. For example, Rg1 can decrease ROS production and further increase the ratio of Bcl-2/Bax in the radiation-induced HSC mitochondrial apoptosis (Figure 6).

Furthermore, Rg1 may inhibit some key genes in the p16^{INK4a}-Rb, p53-p21$^{Cip/Waf1}$, and SIRT6/NF-κB signaling pathways to protect against HSC aging induced by D-gal, t-BHP, and radiation. The mechanism involves reducing DNA damage, regulating the cell cycle, adjusting telomerase activity, and compensating for the HSC telomere length [42, 45–47].

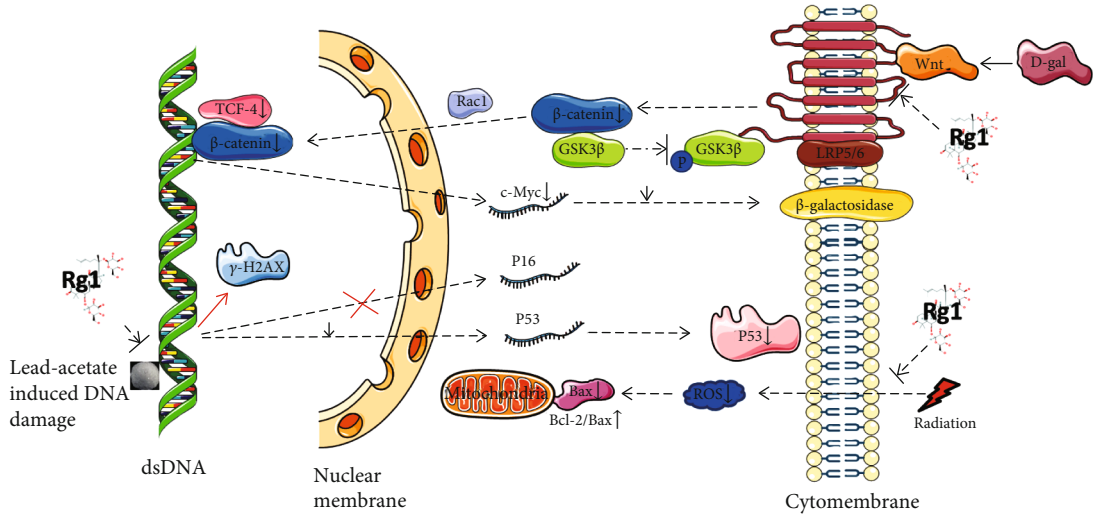


Figure 6: Rg1 alleviates HSC aging by regulating the Wnt/β-catenin and p53 signaling pathways. D-gal: D-galactose; LRP: low-density lipoprotein receptor-related protein; GSK-3β: glycogen synthase kinase 3 beta; Rac1: Ras-related C3 botulinum toxin substrate 1; TCF-1: transcription factor 4.

5. Overview of the Regulation of HPCs and Mature Blood Cells by Rg1

After HSCs differentiate into multipotent progenitors (MPPs), they develop into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), the classic pathway for the differentiation of HPCs [48].

Ginseng extract affects immune cell functions differently according to the specific ginsenoside profile, and the immunomodulatory effects of various ginsenoside monomers are different [49]. For example, Rg1 inhibits TNF-α expression in THP-1 human leukemia cells, whereas the ginsenoside Rh1 increases TNF-α expression [50]. An Rg1/Rb1 mixture and Rg1 exert different effects on IL-6 and TNF-α [49, 51]. These effects are also exhibited by the effect of total ginsenosides or Rg1 monomers on dendritic cells (DCs). Total saponins in ginseng roots can inhibit the maturation of DCs in the presence of lipopolysaccharide (LPS) [51]. However, 10 μg/ml Rg1 can increase CD83, CD80, and HLA-DR expression, reduce CD14 expression in DCs derived from human peripheral blood mononuclear cells, and induce DCs to secrete cytokines (IL-6, TNF-α, and IL-1β) and chemokines (such as IL-8 and IP-10) [52]. Rg1 can stimulate the proliferation of human granulocyte-macrophage progenitors (GMPs) [53]. GMPs can develop into monocytes and myeloblasts. In LPS-activated macrophages, 10 μM Rg1 can also increase the TNF-α levels and decrease the IL-6 protein levels, and these effects are related to regulation of the NF-κB and PI3K/Akt/mTOR pathways [54]. Moreover, 50 μM Rg1 can inhibit RAW264.7 macrophage apoptosis induced by serum deprivation by activating autophagy, and the AMPK/mTOR pathway is one of the signaling pathways associated with the antiapoptotic effects of Rg1 [55]. Rg1 has no obvious effect on megakaryocytes in the spleen of CY-induced mice [27] but can inhibit platelet activation by inhibiting the ERK pathway and attenuate arterial thrombosis [56]. In addition, Rg1 can reduce the infiltration of eosinophils and mast cells in a mouse model of allergic rhinitis [57].

CLPs comprise another important branch of developing HPCs that have the potential to differentiate and develop into T cells, B cells, and natural killer (NK) cells [58]. Rg1 increases the proportion of T helper (Th) cells among total T cells and increases NK cell activity in the mouse spleen [5]. Specifically, Rg1 directly enhances the Th cell response without the participation of antigen-presenting cells (APCs) by increasing the IL-4 and IL-2 levels and reducing the IFN-γ levels to reduce the T helper type 1 (Th1) cell population and increase the T helper type 2 (Th2) cell population in the spleen [59]. In a murine sepsis model, Rg1 increases the neutrophil count in the abdominal cavity and inhibits lymphocyte apoptosis in the thymus and spleen [60].

In total, various in vitro cell stimulation experiments and in vivo animal disease models have shown that Rg1 could regulate the development of myeloid and lymphoid progenitor cells and affect the activity and secretion of mature blood cells, and the progeny of HPCs has also been shown to regulate HPC behavior, which suggests that Rg1 may effectively regulate both innate and adaptive immunity (Figure 7).

6. Rg1 May Indirectly Regulate the HSC Niche

Niches with various functions exist in different areas of the bone marrow. For example, the endosteal niche can support the quiescence and maintenance of HSCs, whereas the arteriolar niche maintains quiescent HSCs, and the sinusoidal niche supports the cycling of HSCs [61, 62]. New studies have also indicated that HSCs in perisinusoidal niches are protected from aging [63]. Moreover, MSCs, the vasculature, and nerve fibers can maintain quiescent HSCs and/or control HPC differentiation through cell-to-cell communication within the niche [64]. Importantly, Rg1 may be able to

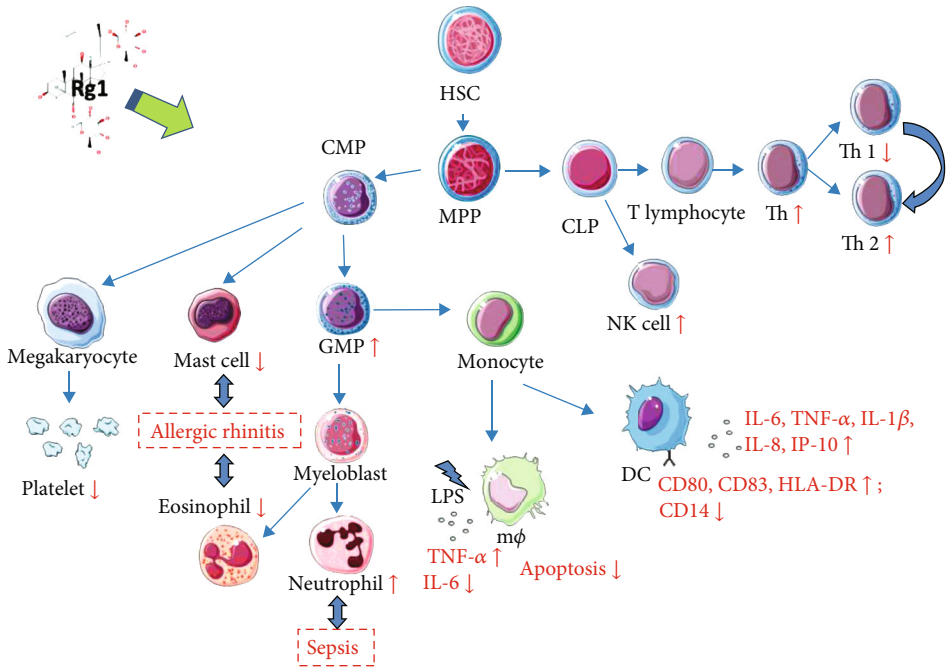


Figure 7: Rg1 regulates HPCs and mature blood cells in the myeloid/lymphoid cell lineage. HSC: hematopoietic stem cell; MPP: multipotent progenitor; CMP: common myeloid progenitor; GMP: granulocyte-macrophage progenitor; LPS: lipopolysaccharide; mφ: macrophage; DC: dendritic cell; CLP: common lymphoid progenitor; NK: natural killer cell; Th: T helper.

regulate MSCs, endothelial cells, nerve cells, and other cells in the niche to support HSC quiescence or development.

MSCs are critical niche constituents of the bone marrow and are major contributors to many currently known niche factors, such as CXCL12, SDF, and IL-7 [65]. Rg1 can effectively regulate MSC proliferation, differentiation, senescence, and apoptosis [17]. Furthermore, studies on bone marrow MSCs in aging D-gal rats have shown that Rg1 could directly enhance the antioxidant and anti-inflammatory capabilities of bone marrow MSCs, improve the microenvironment, and further prevent HSC senescence [30, 66]. These results show that Rg1 could prevent HSC senescence by regulating MSCs in the bone marrow niche.

Vascular endothelial cells play roles in supporting the transport of HSCs [67], and endothelial-related signals (e.g., Notch ligands and E-selectin) might regulate HSC expansion and bone marrow hematopoiesis after myelosuppressive stress [68]. Rg1 can induce vascular endothelial growth factor expression in human endothelial cells and promote proliferation, migration, adhesion, and vasculogenesis in vitro [69, 70]. These results indicate that Rg1 may expand HSCs by regulating endothelial cells in the HSC niche.

HSCs mostly exist in a state of quiescence, and alterations in the metabolism of quiescent HSCs help these cells survive for extended periods of time in hypoxic environments [71]. The stimulation of quiescent HSCs by cell damage initiates active division. The dysregulation of these transitions can lead to stem cell exhaustion or the gradual loss of active HSCs. Studies have shown that the adrenergic nerves of the sympathetic nervous system mobilize HSCs and promote the recovery of hematopoietic function in the

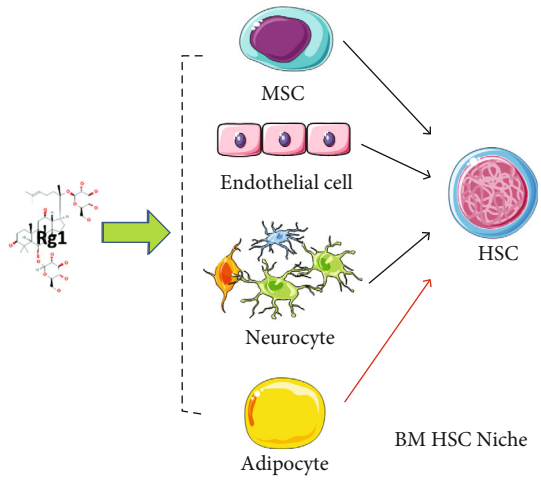


Figure 8: Rg1 may regulate MSCs, endothelial cells, neurocytes, and adipocytes in the bone marrow niche and thus indirectly maintain HSCs.

niche; moreover, adrenergic nerve-related Schwann cells may contribute to the quiescence of HSCs through TGF-β signaling [72, 73]. Rg1 promotes the proliferation of primary Schwann cells and the expression of neurotrophic factors while supporting the resistance of these cells to hydrogen peroxide-induced oxidative damage [74, 75]. This finding suggests that Rg1 may maintain quiescent HSCs by regulating Schwann cells.

Bone marrow adipocytes can act as negative regulators of the hematopoietic microenvironment [76]. Rg1 inhibits the development and maturation of adipocytes by activating C/EBP homologous protein 10 in 3T3-L1 cells [77]. This

finding suggests that Rg1 may also antagonize bone marrow adipogenesis and thereby benefits the hematopoietic microenvironment and protects HSCs.

In summary, in the bone marrow niche, Rg1 may alleviate HSC senescence through MSCs, regulate endothelial cells to expand HSCs, activate Schwann cells to maintain quiescent HSCs, and protect HSCs by inhibiting the formation of adipocytes (Figure 8). However, the relevant direct evidence must be verified by experimental data.

7. Conclusions and Remarks

Through the use of bioinformatics and molecular docking methods to analyze the molecular pharmacological mechanism through which Rg1 regulates HSCs/HPCs, we predicted that GVHD is a possible disease target of Rg1 therapy and that ACE is a potential target protein through which Rg1 regulates the proliferation and differentiation of HSCs/HPCs. A review of the literature also showed that Rg1 may regulate HSC proliferation and can activate extramedullary HSCs to migrate to the bone marrow. These results suggest a new strategy for HSC expansion in vitro and a new method for improving the HSC homing rate and alleviating GHVD in HSC transplantation in vivo. Moreover, the ability of Rg1 to alleviate HSC aging and regulate HPC development suggests that Rg1 exerts direct effects on the maintenance of HSCs/HPCs. However, whether Rg1 can promote the proliferation of HSCs without affecting their differentiation in vitro and whether Rg1 can enhance the HSC homing rate while reducing GVHD during HSC transplantation in vivo are worth further comprehensive exploration.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Fang He and Guanping Yao share first authorship.

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Supplementary Materials

Supplementary Material 1: putative targets of Rg1 identified using TargetNet. Supplementary Material 2: putative targets of Rg1 identified using SwissTargetPrediction. Supplementary Material 3: genes involved in HSC proliferation. Supplementary Material 4: genes involved in HSC migration. Supplementary Material 5: genes involved in HSC differentiation. Supplementary Material 6: genes involved in HPC differentiation. Supplementary Material 7: databases used in this study. *(Supplementary Materials)*

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Research Article

Sox9-Increased miR-322-5p Facilitates BMP2-Induced Chondrogenic Differentiation by Targeting Smad7 in Mesenchymal Stem Cells

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Bone morphogenetic protein 2 (BMP2) induces effective chondrogenesis of mesenchymal stem cells (MSCs) by promoting Sox9 expression. However, BMP2 also induces chondrocyte hypertrophy and endochondral ossification by upregulating Smad7 expression, which leads to the disruption of chondrogenesis. In addition, Smad7 can be inhibited by Sox9. Therefore, the underlying mechanism is not clear. Currently, an increasing number of studies have shown that microRNAs play a pivotal role in chondrogenic and pathophysiological processes of cartilage. The purpose of this study was to determine which microRNA is increased by Sox9 and targets Smad7, thus assisting BMP2 in maintaining stable chondrogenesis. We found that miR-322-5p meets the requirement through next-generation sequencing (NGS) and bioinformatic analysis. The targeting relationship between miR-322-5p and Smad7 was confirmed by dual-luciferase reporter assays, qPCR, and western blotting (WB). The in vitro study indicated that overexpression of miR-322-5p significantly inhibited Smad7 expression, thus causing increased chondrogenic differentiation and decreased hypertrophic differentiation, while silencing of miR-322-5p led to the opposite results. Flow cytometry (FCM) analysis indicated that overexpression of miR-322-5p significantly decreased the rate of early apoptosis in BMP2-stimulated MSCs, while silencing of miR-322-5p increased the rate. A mouse limb explant assay revealed that the expression of miR-322-5p was negatively correlated with the length of the BMP2-stimulated hypertrophic zone of the growth plate. An in vivo study also confirmed that miR-322-5p assisted BMP2 in chondrogenic differentiation. Taken together, our results suggested that Sox9-increased miR-322-5p expression can promote BMP2-induced chondrogenesis by targeting Smad7, which can be exploited for effective tissue engineering of cartilage.

1. Introduction

Traumatic or degenerative cartilage defects are a challenging clinical issue, as cartilage tissue is devoid of vascular, neural, or lymphatic structures [1]. The above characteristics contribute to the poor self-healing capacity of cartilage. Thus, cartilage needs to be reestablished once injured [2]. Mesenchymal stem cells (MSCs) have been identified as ideal seed cells in cartilage tissue engineering due to their chondrogenic differentiation potential [2–4].

Bone morphogenetic protein 2 (BMP2), a member of the transforming growth factor beta (TGF-β) superfamily, is a potent growth factor for the induction of MSC chondrogenic differentiation [5–7]. However, BMP2 alone cannot achieve stable chondrogenesis, as it stimulates chondrogenic hypertrophic differentiation and endochondral ossification, which

destroy the cartilage phenotype [3, 8, 9]. Sox9, induced significantly by BMP2, is the key transcription factor that maintains the chondrocyte phenotype and cartilage homeostasis. This molecule plays an important role in the production and protection of the extracellular matrix of articular cartilage [10–13]. A previous study by our team showed that overexpression of Sox9 enhances BMP-2-induced chondrogenic differentiation in MSCs by downregulating Smad7 expression [14–16]. However, the underlying mechanism of how Sox9 regulates Smad7 is not clear.

Increasing evidence indicates that microRNAs (miRNAs) are crucial for the regulatory network in chondrocyte differentiation and cartilage function [17, 18]. miRNAs are a class of noncoding, single-stranded, and small-molecule RNAs that are approximately 18–24 nucleotides in length. They play a crucial role in many biological processes through posttranscriptional negative regulation of target gene expression by sequence-specific binding to the 3′ untranslated regions (UTRs) of their target messenger RNAs (mRNAs) [17, 19, 20]. Therefore, we hypothesized that Sox9 could promote the expression of certain miRNAs that target and inhibit Smad7 expression.

In the present study, we investigated the function of miR-322-5p in BMP2-mediated chondrogenic and hypertrophic differentiation in MSCs. Sox9 was found to increase the expression of miR-322-5p, which targeted Smad7. Our experiments revealed that overexpression of miR-322-5p suppressed BMP2-induced MSC early apoptosis and chondrocyte hypertrophy, thus facilitating BMP2-induced chondrogenic differentiation. These findings help elucidate BMP2-mediated chondrogenic and hypertrophic differentiation, which can be exploited for BMP2-mediated cartilage tissue engineering.

2. Materials and Methods

2.1. Cell Culture and Chemicals. Mouse bone marrow MSC C3H10T1/2 and human embryonic kidney (HEK) 293 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). Cell lines were maintained in complete Dulbecco's modified Eagle's medium (DMEM, BioExplorer, USA) supplemented with 10% fetal bovine serum (FBS, PAN Biotech, Germany), 100 mg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2). Unless mentioned otherwise, all chemicals were purchased from Thermo-Fisher Scientific or Sigma-Aldrich.

2.2. Construction and Generation of Recombinant Adenoviral Vectors AdBMP2, AdSox9, AdGFP, AdshSox9, and AdRFP. AdEasy technology was used to generate recombinant adenoviruses as previously described [21]. AdBMP2, AdSox9, and AdshSox9 were previously characterized [8, 14, 16, 22]; AdGFP and AdRFP were used as mock virus controls. Briefly, the full-length transcript of mouse-derived Sox9 and the coding region of human-derived BMP2 were PCR amplified and subcloned into an adenoviral shuttle vector to generate recombinant adenoviral vectors; vectors containing Sox9 or BMP2 were subsequently used to generate recombinant adenoviruses in HEK-293 cells. AdshSox9 was purchased from Vigene (Shandong, China). For monitoring infection efficiency, AdBMP2 and AdSox9 were flagged with green fluorescent protein (GFP), and AdshSox9 was labeled with red fluorescent protein (RFP).

2.3. Chondrogenic Differentiation of MSCs in Micromass Culture. To mimic the condensation of MSCs, we used micromass culture to induce chondrogenic differentiation as previously described [23]. C3H10T1/2 cells were infected with AdGFP, AdRFP, AdBMP2, AdSox9, or AdshSox9. To manipulate miR-322-5p expression, we infected miR-322-5P agomir and antagomir, purchased from GenePharma (Shanghai, China), by siRNA-Mate™ (GenePharma) according to the manufacturer's instructions, using agomir-NC or antagomir-NC as normal controls, respectively. Twenty-four hours after infection, the cells were collected, resuspended at a high density (~10^5 per 50 μl of DMEM), subsequently seeded at the center of each well in 6-well plates, and then incubated in a CO_2 incubator. Two hours after incubation, 2 ml of complete DMEM was added to each well; half of the medium was replaced every 3 days.

2.4. RNA Isolation and qPCR. Total RNA was isolated with RNAiso Plus (TaKaRa, China) and subjected to reverse transcription with a PrimeScript RT reagent kit (TaKaRa, China) according to the manufacturer's instructions. The qPCR experiment was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad, United States) using SYBR Premix Ex Taq II kit (TaKaRa, China) under the following conditions: 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s, repeating 40 cycles. GAPDH was used as the internal reference, and data were normalized by the $2^{-\Delta\Delta ct}$ method. The primer sequences are shown in Table 1.

2.5. NGS and Bioinformatic Analysis. Total RNA from both groups, BMP2+RFP and BMP2+shSox9, was extracted for NGS on the Illumina HiSeq2500 sequencer 50 SE at day 6 after adenoviral infection. Then, data were analyzed to generate volcano plots, Venn diagrams, and scatter plots at https://www.omicstudio.cn/tool [24]. Prediction of miRNAs targeting Smad7 was performed in the databases miRanda, StarBase, and TargetScan.

2.6. Dual-Luciferase Reporter Assay. For detection of the interaction between Smad7 and miR-322-5p, plasmids containing mutant-type Smad7-3′UTR (Smad7-3′UTR-MT) and wild-type Smad7-3′UTR (Smad7-3′UTR-WT) were constructed. When HEK-293 cells cultured in 24-well plates reached 80% confluency, 50 nM miR-322-5p agomir or NC was cotransfected into cells with 2 μg plasmids mediated by Lipofectamine 2000 (Invitrogen). Moreover, Renilla luciferase- (RL-) loaded pRL-TK was transfected as an internal control. After 48 h, the Dual-Luciferase Reporter Gene Assay Kit (Beyotime) was used to detect the intensity of RL and firefly luciferase (FL) according to the manufacturer's protocol. Consequently, the ratio of FL to RL reflected the suppressive effect of miR-322-5p on Smad7. Each group had five duplicate wells.

Table 1: The primer sequences used for qRT-PCR.

Genes	Primer sequences (forward: 5′-3′)	Primer sequences (reverse: 5′-3′)
Smad7	AAGATCGGCTGTGGCATC	CCAACAGCGTCCTGGAGT
COL2A1	CAACACAATCCATTGCGAAC	TCTGCCCAGTTCAGGTCTCT
Sox9	AGCTCACCAGACCCTGAGAA	TCCCAGCAATCGTTACCTTC
COL10A1	TGCTGCCCTGGTCTTACTCT	GCCTTGGGATCCTAAACCT
GAPDH	CTACACTGAGGACCAGGTTGTCT	TTGTCATACCAGGAAATGAGCTT

2.7. Western Blotting (WB). Protein extraction was performed with lysis buffer, radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, China) containing 1% phenylmethanesulfonyl fluoride (PMSF) (Beyotime Biotechnology, China), and subsequent sonication. After centrifugation, the supernatant was boiled for denaturation and determination of total protein concentration using the BCA protein assay kit (Beyotime Biotechnology, China). Equivalent amounts of protein were loaded for electrophoresis on 7-10% SDS-PAGE gels (Omni-Easy™ One-Step PAGE Gel Fast Preparation Kit, EpiZyme, China) and transferred to polyvinylidene fluoride membranes (PVDF, 0.2 μm, Bio-Rad). After the membranes were blocked with 5% skim milk at room temperature for 1 h, proteins were incubated overnight at 4°C with the following primary antibodies: Sox9 (Zen Bio, 1 : 2000), COL2A1 (Abcam, 1 : 3000), COL10A1 (Santa Cruz Biotechnology; 1 : 1000), Smad7 (Santa Cruz,1 : 1000), and GAPDH (Zen Bio, 1 : 2000). After the membranes were washed with TBST, they were incubated with the corresponding secondary antibodies (goat anti-rabbit IgG, 1 : 10000, Zen Bio) for 1 h at room temperature. Following sequential washing with TBST and TBS, the target proteins were detected by an ECL detection kit (Thermo Fisher Scientific), and ImageJ software was used for quantification of band density.

2.8. Apoptosis Detection by Flow Cytometry. Cells were treated with trypsin (0.25%) and monitored under a microscope throughout the process of digestion, which was immediately stopped by adding DMEM containing FBS when most of the cells became round. After centrifugation, the cells were repeatedly resuspended in PBS gently and centrifuged twice, followed by resuspension in 500 μl of PBS for immediate detection on CytoFLEX.

2.9. Mouse Fetal Limb Explant Culture. Forelimbs of mouse embryos (E18.5) were dissected of skin and most soft tissue, except periosteum, under sterile conditions and incubated in DMEM containing 0.5% bovine serum albumin (BSA, Sigma), 50 μg/ml ascorbic acid, 1 mM β-glycerophosphate, 100 mg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2) for up to 14 days as previously described [25, 26]. Five samples were cultured in each well. Upon the initiation of incubation, the skin-free limbs were infected by adding AdBMP2, AdSox9 or AdshSox9, miR-322-5P agomir, or antagomir to the culture medium. Half of the medium was changed every second day. For monitoring the survival of the cells in the forelimbs, GFP and RFP signals were observed under a microscope. The tissues were fixed for histological evaluation after 14 days of culturing.

2.10. Subcutaneous MSC Implantation. The animal use and care and experimental procedures were approved by the Chongqing Medical University Animal Care and Use Committee. The subcutaneous stem cell implantation procedure was carried out as described [3, 14]. Briefly, C3H10T1/2 cells were infected with AdBMP2, AdGFP, AdRFP, AdSox9, AdshSox9, miR-322-5P agomir, and antagomir. Twenty-four hours after transfection, the cells were collected and resuspended in PBS-diluted Matrigel (Corning) for subcutaneous injection into the flanks of athymic nude mice (4 weeks old, female, $n = 3$/group, $4 * 10^6$ cells per injection). Four weeks after injection, the animals were sacrificed for collection of ectopic masses. Following fixation in 4% paraformaldehyde (Servicebio, Wuhan, China) for 24 h at room temperature, the masses were subjected to ethylenediaminetetraacetic acid (EDTA) for decalcification at 4°C for 14 days, followed by embedding in paraffin. Serial 5 μm thick sections were processed for special staining and histological evaluations.

2.11. Histological Evaluation: Hematoxylin and Eosin (H&E), Alcian Blue Staining, and Masson's Trichrome. After sequential deparaffinization with xylene and rehydration with graded ethanol, H&E, Masson's trichrome, and Alcian Blue staining was performed using a standard protocol as described previously [8, 14, 16]. Briefly, the deparaffinized samples were first subjected to antigen retrieval and fixation, followed by H&E and Masson's trichrome staining. A light microscope (Olympus, Japan) was used for histological evaluation.

2.12. Immunohistochemistry Assay. After sequential deparaffinization with xylene and rehydration with graded ethanol, sections were boiled in 10 mM citrate buffer at 95–100°C for 10 min for antigen retrieval, rinsed in 3% H_2O_2 at room temperature for 10 min to inhibit endogenous peroxidase activity, and blocked with 10% goat serum at room temperature for 10 min. Then, the sections were incubated with primary antibodies against collagen 2α1 (COL2A1) (Abcam, 1 : 400) and collagen 10α1 (COL10A1) (Abcam, 1 : 200) at 4°C overnight. After washing, the sections were incubated with secondary antibody at 37°C for 30 min, followed by incubation with streptavidin–HRP conjugate for 20 min at room temperature. Staining without primary antibody was used as a negative control. A microscope (Olympus, Japan) was used for imaging.

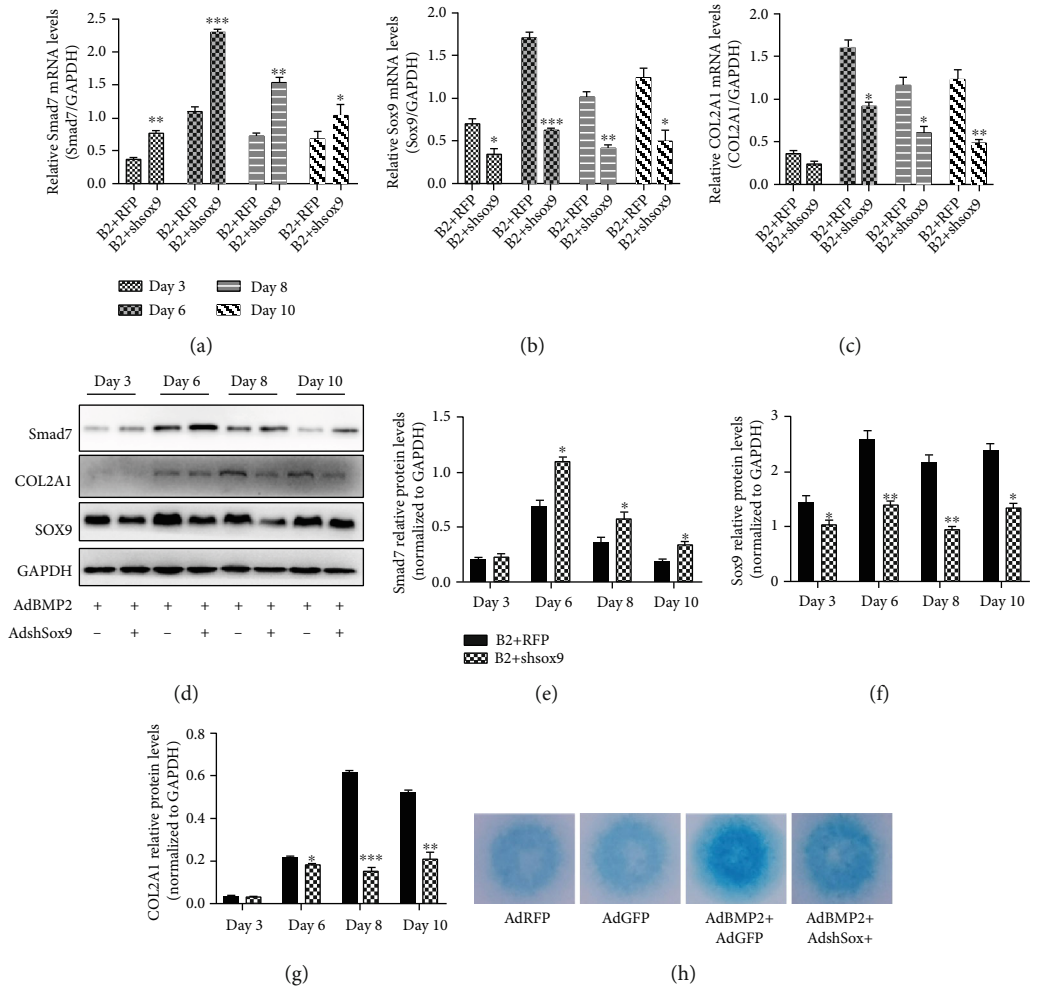


Figure 1: Silencing Sox9 inhibited BMP2-induced chondrogenesis. (a–c) Gene expression detected by qPCR revealed that silencing Sox9 inhibited BMP2-induced expression of Sox9 and COL2A1, while it promoted that of Smad7. (d–g) WB mimicked the results of qPCR. (h) Alcian Blue staining of micromasses demonstrated weakened chondrogenic capacity of shSox9-treated MSCs. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, comparison with group AdBMP2 at corresponding time points. B2: BMP2.

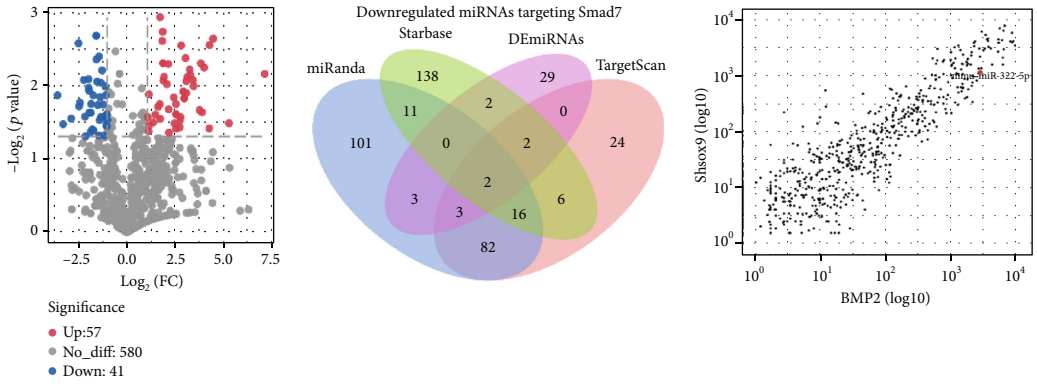
2.13. Statistical Analysis. All experiments were performed at least three times independently. Data are expressed as the mean ± standard deviation (SD) and were analyzed with SPSS software (Version 21, IBM). Statistical analyses were conducted using one-way analysis of variance and Student's *t*-test; $p < 0.05$ was considered statistically significant.

3. Results

3.1. Silencing Sox9 Inhibited BMP2-Induced Chondrogenesis. AdBMP2 was infected in C3H10T1/2 cells with or without AdshSox9. Gene expression detected by qPCR at days 3, 6, 8, and 10 after infection revealed that silencing Sox9 inhibited BMP2-induced Sox9 and COL2A1 expression while promoting BMP2-induced Smad7 expression (Figures 1(a)–1(c)). The WB results were consistent with the results of qPCR (Figures 1(d)–1(g)). Micromasses infected with AdRFP, AdGFP, AdBMP2, and AdBMP2+AdshSox9 were cultured for 7 days before being subjected to Alcian Blue staining, which demonstrated weakened staining of the AdBMP2+AdshSox9 group compared with the AdBMP2+AdRFP group. These results indicated that silencing Sox9 inhibited BMP2-induced chondrogenesis.

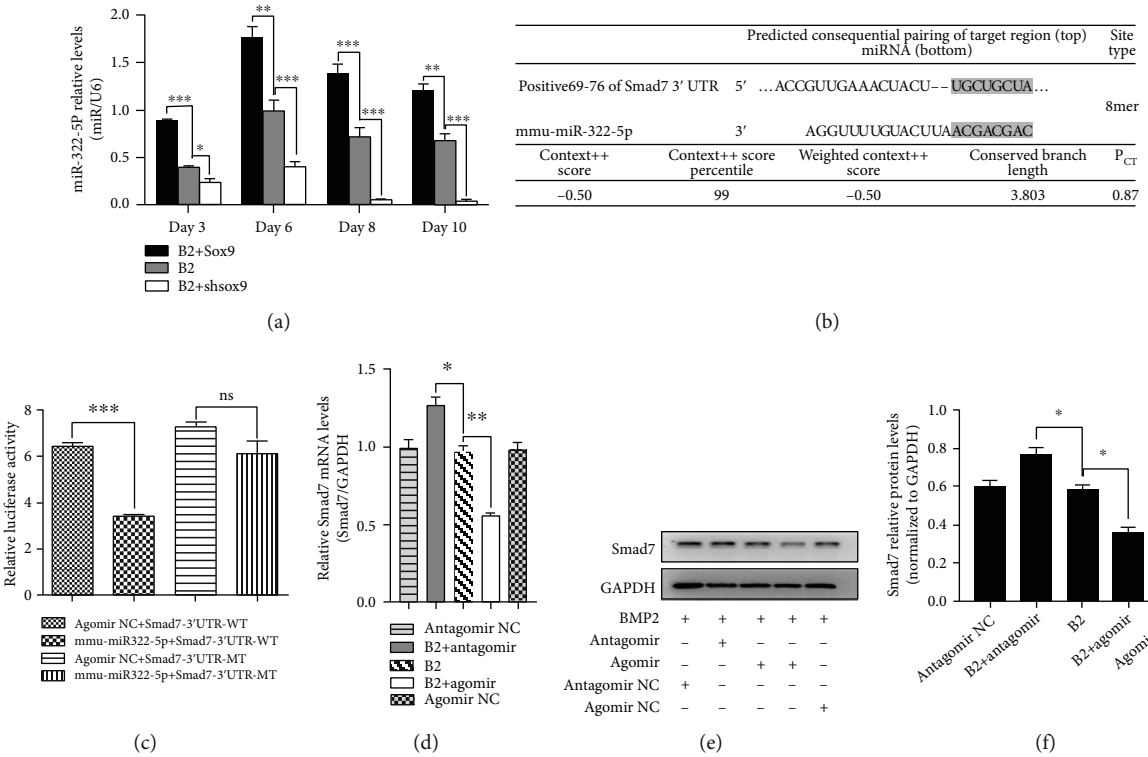
3.2. NGS and Bioinformatic Analyses Demonstrated That Silencing of Sox9 Decreased the Expression of miR-322-5p, Which Was Predicted to Target Smad7. The volcano plot (Figure 2(a)) showed that 41 and 57 miRNAs had downregulated and upregulated expression, respectively, in the BMP2+shSox9 group. After prediction for miRNAs targeting Smad7 in the databases miRanda, StarBase, and TargetScan, Venn diagram analysis revealed that only two miRNAs among the miRNAs with downregulated expression in the BMP2+shSox9 group appeared in all three databases (Figure 2(b)). Consequently, miR-322-5p, but not miR-181-5p, was the focus of the following experiments. Next, the scatter plot showed that the correlation coefficient was 0.9, which suggested a reliable trend between the groups (Figure 2(c)).

3.3. The Sox9/miR-322-5p/Smad7 Axis Was Confirmed by qPCR, WB, and Dual-Luciferase Reporter Assays. Total RNA extraction at days 3, 6, 8, and 10 postinfection was



(a) (b) (c)

Figure 2: The NGS and bioinformatic analysis demonstrated that silencing of Sox9 decreased the expression of miR-322-5p which was predicted to target Smad7. (a) The volcano plot showed the upregulated and downregulated miRNAs in the BMP2+shSox9 group. (b) The Venn diagram analysis revealed that only two downregulated miRNAs, including miR-322-5p, appeared in all three databases. (c) The correlation coefficient between groups was 0.9, and miR-322-5p was marked. FC: fold change.



(a) (b) (c) (d) (e) (f)

Figure 3: The Sox9/miR-322-5p/Smad7 axis was confirmed by qPCR, WB, and dual-luciferase reporter assay. (a) Relative expression levels of miR-322-5p among groups at different time points. (b) Predicted consequential pairing of Smad7 and miR-322-5p. (c) Dual-luciferase reporter assay confirmed the targeting relationship between Smad7 and miR-322-5p. (d) qPCR revealed the silencing effect of miR-322-5p on Smad7. (e, f) Results of WB were consistent with those of qPCR. Agomir represents mimics of miR-322-5p; antagomir represents blocker of miR-322-5p. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$. NC: negative control; B2: BMP2.

performed on C3H10T1/2 cells infected with AdBMP2+-AdSox9, AdBMP2, and AdBMP2+AdshSox9. Subsequently, the expression of miR-322-5p was detected by qPCR, which suggested an increased response by overexpressing Sox9 and a downregulated response by silencing Sox9 (Figure 3(a)). According to the prediction on TargetScan, miR-322-5p matched the position 69-76 of the Smad7 3′UTR

Table 2: The sequences used for miR-322-5p function regulation.

Genes	Primer sequences (5′ to 3′)
mmu-miR-322-5p agomir	CAGCAGCAAUUCAUGUUUUGGA CAAAACAUGAAUUGCUGCUCUU
mmu-miR-322-5p antagomir	UCCAAAACAUGAAUUGCUGCUG

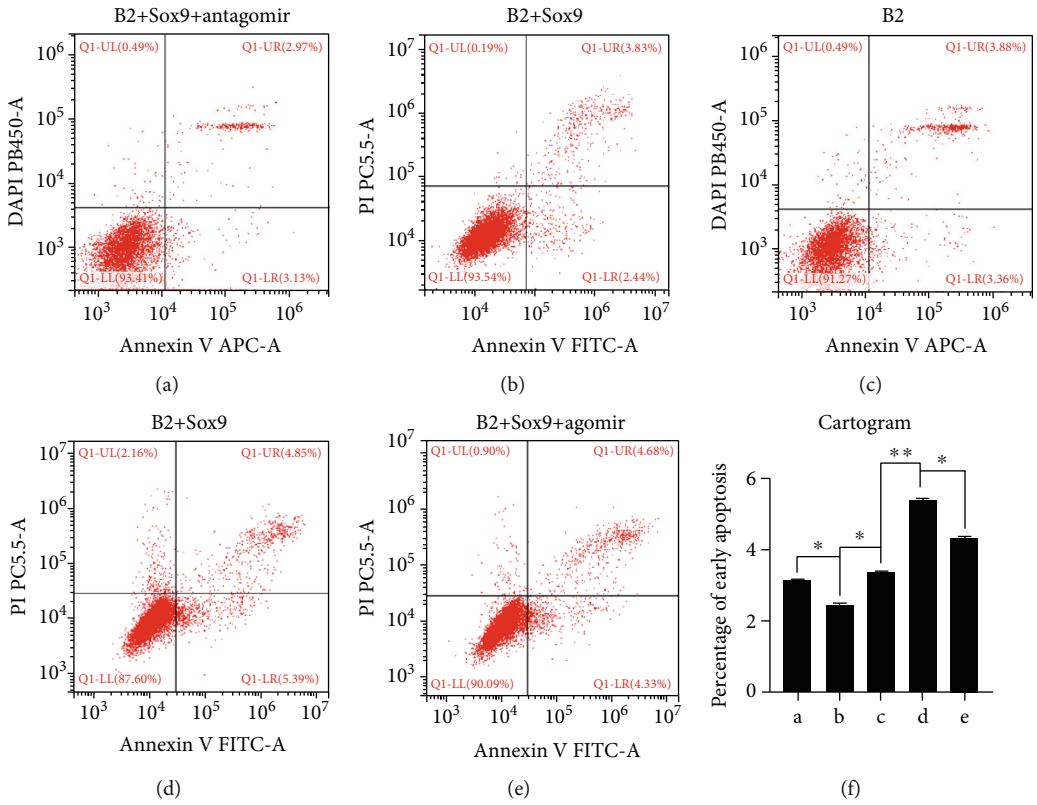


FIGURE 4: Flow cytometry was performed for detection of early apoptosis. (a–e) The percentage of early apoptosis in each group was shown. (f) $^{*}p < 0.05$ and $^{**}p < 0.01$. B2: BMP2.

(Figure 3(b)) [27]. To verify the targeting relationship between miR-322-5p and Smad7, we used antagomirs and agomirs to promote and inhibit miR-322-5p functions, respectively. The sequences are shown in Table 2. Given the expression of BMP2, WB results, in accordance with the qPCR results, showed decreased expression of Smad7 upon miR-322-5p agomir transfection and increased expression of Smad7 upon miR-322-5p antagomir transfection (Figures 3(c)–3(e)). Furthermore, a dual-luciferase reporter assay was performed and demonstrated significantly lower luciferase activity in the mmu-miR-322-5p+Samd7-3′UTR-WT group than in the agomir NC+Smad7-3′UTR-WT group; moreover, no significant difference was observed between the mmu-miR-322-5p+Samd7-3′UTR-MT and agomir NC+Smad7-3′UTR-MT groups (Figure 3(f)).

3.4. Smad7-Induced Early Apoptosis Was Negatively Correlated with the Expression Level of miR-322-5p. To determine the effect of miR-322-5p on Smad7-related early apoptosis, we performed flow cytometry (FCM). As indicated by the results, overexpression of Sox9 obviously decreased the early apoptosis rate (Figures 4(b) and 4(c)), which was partly restored by forced expression of miR-322-5p antagomir (Figures 4(a) and 4(b)); moreover, silencing of Sox9 remarkably upregulated the early apoptosis rate (Figures 4(c) and 4(d)), which was partially reversed by use of the miR-322-5p agomir (Figures 4(d) and 4(e)). The cartogram shows the early apoptosis rate in each group (Figure 4(f)).

3.5. BMP2-Induced Chondrocyte Hypertrophy in Fetal Mouse Forelimb Explants Was Inhibited by miR-322-5p. After culture for 14 days, fetal mouse forelimbs were subjected to sectioning and H&E staining to evaluate the length of the hypertrophic zone. The results demonstrated that overexpressing Sox9 reduced the BMP2-induced hypertrophic zone, which was partly reversed by forced expression of the miR-322-5p antagomir (Figures 5(a)–5(c)); moreover, silencing of Sox9 extended the hypertrophic zone, which was partly reversed by the use of the miR-322-5p agomir (Figures 5(c)–5(e)). The arrows in dark blue and yellow represent the lengths of the prehypertrophic and hypertrophic zones (HZs), respectively (Figure 5(f)). The cartogram shows the length of the HZ in each group (Figure 5(g)).

3.6. BMP2-Induced Chondrogenesis Was Enhanced by miR-322-5p In Vitro. C3H10T1/2 cells infected with AdBMP2+AdSox9+antagomir, AdBMP2+AdSox9, AdBMP2, AdBMP2+AdshSox9, or AdBMP2+AdshSox9+agomir were cultured in micromasses for 7 days, followed by total protein extraction and Alcian Blue staining. As shown by the blots, overexpressing Sox9 promoted BMP2-induced expression of COL2A1 but inhibited that of COL10A1 and Smad7 (Figures 6(a)–6(d)). When Sox9-induced miR-322-5p expression was silenced by antagomir, the expression of all the markers above was partly reversed (Figures 6(a)–6(d)). However, silencing Sox9 inhibited BMP2-induced expression of COL2A1 but promoted that of COL10A1 and Smad7 (Figures 6(a)–6(d)). When miR-

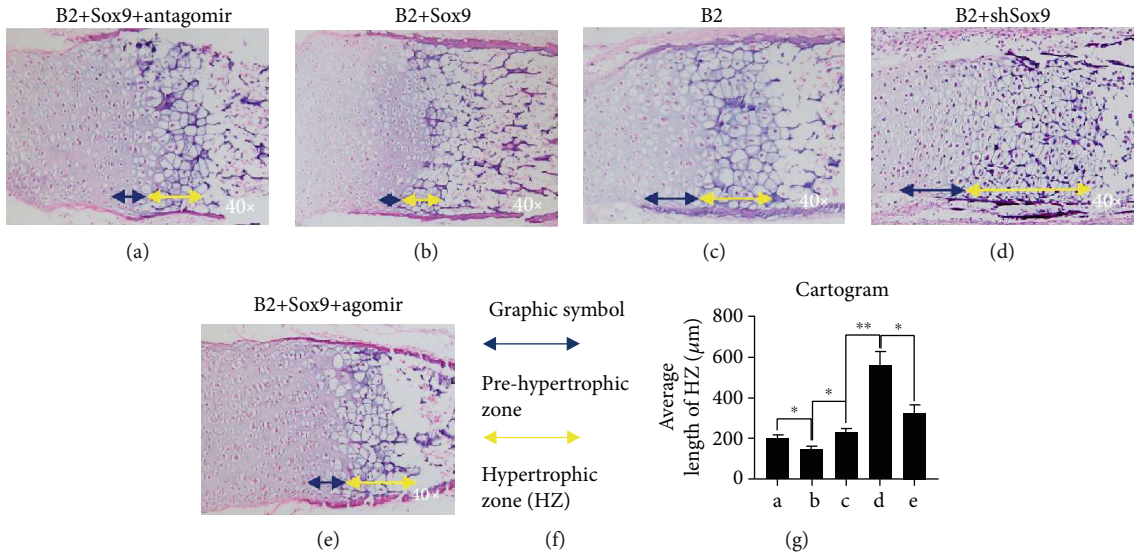


Figure 5: BMP2-induced chondrocyte hypertrophy in fetal mouse forelimb explant was inhibited by miR-322-5p. (a–e) Forelimbs were subjected to H&E staining for histological evaluation. (f) The arrows in dark blue and yellow represent the length of prehypertrophic and hypertrophic zone (HZ), respectively. (g) $^{*}p < 0.05$; $^{**}p < 0.01$. B2: BMP2.

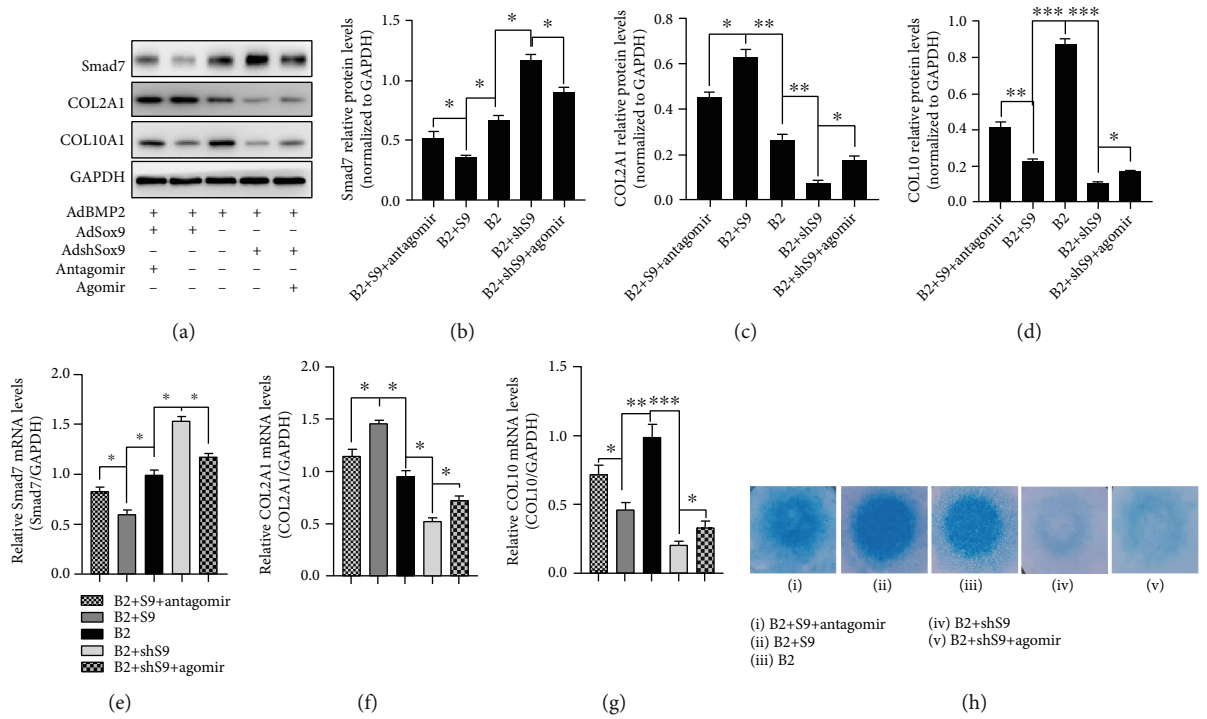


Figure 6: BMP2-induced chondrogenesis was enhanced by miR-322-5p. (a–d) WB revealed that overexpression of miR-322-5p inhibited expression of Smad7 and COL10A1 and promoted that of COL2A1; silencing of miR-322-5p brought the opposite changes. (e–g) Results of qPCR mimicked those of WB. (h) Alcian Blue staining supported the effect of miR-322-5p in facilitating BMP2-induced chondrogenesis. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$. B2: BMP2; S9: Sox9; shS9: shSox9.

322-5p expression was induced by using agomir, the results were also partly reversed (Figures 6(a)–6(d)). In addition, expression at the transcriptional level supported the results of WB analysis (Figures 6(e)–6(g)). Furthermore, Alcian Blue staining suggested that the Sox9-enhanced staining was weakened by the antagomir (Figure 6(h) i–iii); moreover, the shSox9-weakened staining was enhanced by the use of the miR-322-5p agomir (Figure 6(h) iii–v).

3.7. BMP2-Induced Chondrogenesis Was Enhanced by miR-322-5p In Vivo. For determination of whether miR-322-5p is effective in silencing Smad7, thus facilitating BMP2-

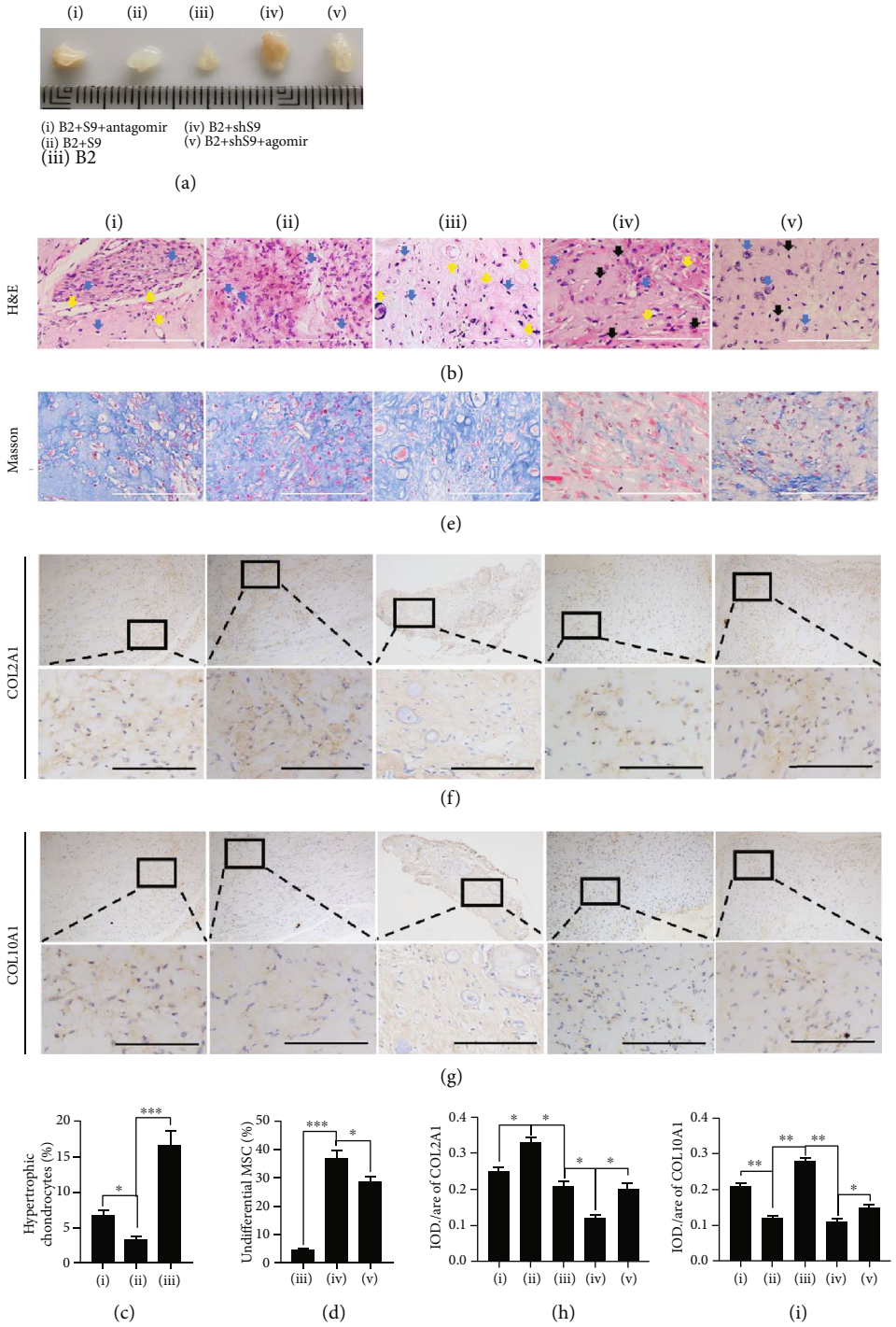


Figure 7: BMP2-induced chondrogenesis was enhanced, and chondrocyte hypertrophy was weakened by miR-322-5p in vivo. (a) Masses were retrieved after 4 weeks of injection. (b) Overexpressing Sox9 decreased the number of hypertrophic chondrocytes (yellow arrow), which was partly reversed by the use of the miR-322-5p antagomir ((b) i–iii); moreover, silencing Sox9 decreased the number of differentiated chondrocytes (blue arrow) and increased the number of undifferentiated MSCs (dark arrow), which was partly reversed by forced expression of the miR-322-5p agomir ((b) iii–v). (c, d) Quantitative analysis of randomized three fields in each group. (e) According to the Masson staining, increased formation of cartilage tissue promoted by Sox9 was partly reversed by adding of miR-322-5p antagomir, while decreased production of cartilage tissue caused by silencing Sox9 was partly reversed by forced expression of miR-322-5p agomir. (f, g) IHC showed that overexpression of Sox9 increased the synthesis of COL2A1 and decreased the generation of COL10A1, and both were partly reversed by use of miR-322-5p antagomir; simultaneously, silence of Sox9 decreased the production of COL2A1 and increased the formation of COL10A1, and both were partly reversed by use of miR-322-5p agomir. (h, i) Quantitative analysis of positive-stained area. Integral optical density/area (IOD/area) was calculated with Image Pro Plus software. Scale bar = 150 μm; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

induced chondrogenesis in vivo, C3H10T1/2 cells infected with AdBMP2+AdSox9+antagomir, AdBMP2+AdSox9, AdBMP2, AdBMP2+AdshSox9, AdBMP2+AdshSox9+agomir, AdGFP, AdRFP, AdSox9, AdSmad7, miR-322-5P agomir, and antagomir were subcutaneously injected into the flanks of nude mice. The results indicated that no detectable masses were formed in the cells infected with AdGFP, AdRFP, AdSox9, miR-322-5P agomir, or antagomir alone. Masses were retrieved after 4 weeks of injection. The amount of Sox9 and miR-322-5p was positively correlated with hyaline cartilage-like appearance (Figure 7(a)).

Based on the histological evaluation, overexpressing Sox9 decreased the number of hypertrophic chondrocytes (yellow arrow), which was partly reversed by the use of the miR-322-5p antagomir (Figures 7(b) i–iii and 7(c)); moreover, silencing Sox9 decreased the number of differentiated chondrocytes (blue arrow) and increased the number of undifferentiated MSCs (dark arrow), which was partly reversed by forced expression of the miR-322-5p agomir (Figures 7(b) iii–v and 7(d)). According to Masson staining, the increased formation of cartilage tissue promoted by Sox9 was partly reversed by the addition of the miR-322-5p antagomir (Figure 7(e) i–iii), while the decreased production of cartilage tissue caused by silencing Sox9 was partly reversed by forced expression of the miR-322-5p agomir (Figure 7(e) iii–v).

Furthermore, immunohistochemistry showed that overexpression of Sox9 increased the synthesis of COL2A1 and decreased the generation of COL10A1, and both changes were partly reversed by use of the miR-322-5p antagomir (Figures 7(f), 7(g) i–iii, 7(h), and 7(i)); moreover, silencing of Sox9 decreased the production of COL2A1 and increased the formation of COL10A1, and both changes were partly reversed by use of miR-322-5p agomir (Figures 7(f), 7(g) iii–v, 7(h), and 7(i)).

4. Discussion

Articular cartilage defects caused by trauma or degeneration are increasing yearly with the development of society and changes in people's living habits. However, the repair of defective cartilage is still a challenging issue worldwide. BMP2-induced chondrogenesis of MSCs has been widely accepted as a chondrogenic model in tissue engineering. However, BMP2 induces not only chondrogenesis but also endochondral ossification [8, 14]. Previous studies by our team have shown that overexpression of Sox9 promotes BMP2-induced chondrogenesis and may function through the suppressive effect of Sox9 on Smad7 [14, 16]. The current study clarified that silencing Sox9 weakened BMP2-induced chondrogenesis by upregulating Smad7 expression. The mechanism may be that Sox9 induces miR-322-5p, which binds to the 3′UTR of Smad7 and inhibits its function.

Sox9 is highly increased by BMP2, and it facilitates BMP2-induced chondrogenic differentiation [11, 16, 28]. This molecule directly regulates the production of COL2A1, which is chondrocyte specific [29, 30]. Smad7 is an inhibitor in the Smad family; it plays an inhibitory role in the TGFβ/BMP pathway, which is crucial in the processes of chondrogenesis [31–33]. Previous studies have found that BMP2-induced high expression of Smad7 serves as the key inhibitor of chondrogenic differentiation [32, 34]. In addition, the hypertrophic differentiation of chondrocytes, marked with COL10A1 [15, 35], was caused by Smad7, at least in part, during chondrogenic differentiation [15]. Further research revealed that Smad7 inhibited the formation of cartilaginous tissue induced by BMP2 by suppressing the P38 and Smad1/5/8 pathways [32, 36].

Increasing research has revealed the important roles of miRNAs in the processes of chondrocyte formation and the pathophysiological function of cartilage. In this research, NGS and bioinformatic analysis found that miR-322-5p expression was downregulated by silencing Sox9, and the results were further clarified by qPCR. In addition, overexpression of Sox9 upregulated miR-322-5p expression. These results confirmed that Sox9 upregulates miR-322-5p expression. Moreover, the dual-luciferase reporter assay confirmed the targeting relationship between miR-322-5p and Smad7. Subsequently, in vitro experiments also showed that miR-322-5p could indeed inhibit the expression of Smad7 at the transcriptional and translational levels.

Currently, there are only a few reports on the biological functions of miR-322-5p. A previous report showed that miR-322-5p was involved in cardiac hypertrophy in rats with pulmonary hypertension by targeting IGF-1 [37]. In addition, miR-322-5p is involved in FAM3B-mediated hyperglycemic vascular smooth muscle proliferation and migration [38]. RNA sequencing based on cartilage-derived progenitor and stem cells identified miR-322-5p as one of the core regulatory molecules during the progression of OA [39]. In another RNA sequencing analysis performed by our team, high expression of miR-322-5p was positively correlated with BMP-2-induced chondrogenesis (data not shown). It was reported that overexpression of miR-322-5p activated the TGF-β pathway, which is important in maintaining the homeostasis of articular cartilage [37, 38, 40]. Given that previous reports and data have shown the correlation of miR-322-5p with cartilage homeostasis and chondrogenesis, this molecule was predicted to be more associated with Smad7 [41]. Thus, when the final two miRNAs were screened out, we focused on miR-322-5p for subsequent experiments. To the best of our knowledge, this is the first study to reveal the regulatory effect of miR-322-5p on Smad7 and chondrogenesis.

5. Conclusions

In conclusion, our findings suggested that the Sox9/miR-322-5p/Smad7 regulatory axis exists during BMP2-induced chondrogenesis and that Sox9-increased miR-322-5p can target Smad7, thus inhibiting BMP2-induced chondrocyte hypertrophy and assisting in maintaining stable chondrogenesis.

Data Availability

Data will be available on request.

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

Elucidating the Pivotal Neuroimmunomodulation of Stem Cells in Spinal Cord Injury Repair

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Spinal cord injury (SCI) is a distressing incident with abrupt onset of the motor as well as sensory dysfunction, and most often, the injury occurs as result of high-energy or velocity accidents as well as contact sports and falls in the elderly. The key challenges associated with nerve repair are the lack of self-repair as well as neurotrophic factors and primary and secondary neuronal apoptosis, as well as factors that prevent the regeneration of axons locally. Neurons that survive the initial traumatic damage may be lost due to pathogenic activities like neuroinflammation and apoptosis. Implanted stem cells are capable of differentiating into neural cells that replace injured cells as well as offer local neurotrophic factors that aid neuroprotection, immunomodulation, axonal sprouting, axonal regeneration, and remyelination. At the microenvironment of SCI, stem cells are capable of producing growth factors like brain-derived neurotrophic factor and nerve growth factor which triggers neuronal survival as well as axonal regrowth. Although stem cells have proven to be of therapeutic value in SCI, the major disadvantage of some of the cell types is the risk for tumorigenicity due to the contamination of undifferentiated cells prior to transplantation. Local administration of stem cells via either direct cellular injection into the spinal cord parenchyma or intrathecal administration into the subarachnoid space is currently the best transplantation modality for stem cells during SCI.

1. Introduction

Spinal cord injury (SCI) is a distressing incident with abrupt onset of the motor as well as sensory dysfunction [1, 2]. SCI most often occurs as a result of high-energy or velocity accidents as well as contact sports and falls in the elderly [2, 3]. Initially, SCIs were mostly seen in young patients but the trend is currently increasing in elderly patients resulting in cervical canal stenosis [3]. SCI is often associated with personal losses by the patients and their families as well as substantial societal cost [1, 2]. The injury usually results in damage to autonomic neurons at and below the cord resulting in bowel, bladder, and sexual dysfunctions [1, 2].

Human SCI remains a serious challenge with currently no successful treatments [1, 4]. Nevertheless, surgical intervention and subsequent rehabilitation are the only alternatives for SCI treatment. Furthermore, although methylprednisolone is usually given to patients at the acute stage of injury, a consensus of its usage is still a matter of debate in terms of both safety and effectiveness [1, 5, 6]. Stem cell transplantation may provide an effective treatment for SCI due to the self-renewing and multipotential nature of these cells [7]. Thus, analytical hypotheses to consider in translating stem cell therapies for SCI comprise injury severity; cell type; spinal level such as cervical, thoracic, and lumber levels; cell delivery system, and epicenter and/or perilesional injections.

This review therefore explores the key roles of stem cell transplantation for spinal cord injury repair with a focus on the parameters above with the key focus on the influence that the microenvironment has after stem cell transplantation in SCI. The "boolean logic" was utilized to search for the article on the subject matter. Most of the articles were indexed in PubMed with strict inclusion criteria being the type of cells and the mode of delivery as well as the effectiveness or

success after transplantation. The search terms were functional anatomy of the spine, spinal dynamics, and stem cell transplantation and/or SCI in animal models and humans.

2. Functional Anatomy of the Spine

The human spine is a complex column with a combination of substantial structural support and restrictive motions throughout its 24 articulating vertebrae [8, 9]. The cervical spine is very flexible, strong, and mobile in all directions and thus functions as the sensory unit as well support of the head. It permits the sensory structures of the vision, hearing, and smell to move freely in the sagittal plane and also articulate with the environment in the horizontal plane [9, 10]. The cervical spine is long and slender and therefore susceptible to either minor or major injuries [9]. The cervical spine is often divided into three zones which differ both in structure and in function. These divisions include the suboccipital zone (C1 vertebra), a transitional zone (C2 vertebra), and the typical zone (C–7 vertebrae) [9, 11].

The thoracic vertebrae have vertebral bodies that are joined by intervertebral discs as well as longitudinal ligaments and posterior elements that are joined by zygapophysial joints just like cervical and lumbar vertebrae [9]. In all, there are 12 thoracic vertebrae in the human body [8, 9]. The nerves control motor as well as sensory signals in the upper back, chest, and abdomen [9]. Nevertheless, exceptions of the thoracic vertebra arrangement occur at T1 and at T11 and T12, where the head of the rib completely articulates with the like-numbered vertebrae [9]. The essential role of the lumbar spine is to support the thorax as well as the upper limbs. The lumber spine aids load carrying and transmits the weight of loads to the pelvis and lower limbs [9, 12].

Also, the lumbar spine supports a very little range of movement between the thorax and pelvis [13]. In all, there are 5 lumber vertebrae in the human body [12]. The human sacrum is a huge triangular bone comprising of five separate vertebra that fuse along with the intervening intervertebral discs [13]. The sacrum fuses with four bones: the last lumbar vertebra upwards through a disc space as well as the facet joint complex, the coccyx downwards with a ligamentous attachment and seldomly a bone union, and on either side with the ilium via the sacroiliac joint [13].

3. Spinal Dynamics and Injury

The spinal cord is a modeled cylinder comprising of diverse anisotropic elastic dense tissue, with a fibrous surface lining (meninges), suspended in fluid, tethered via small ligaments, and having an intermittent motion as well as tissue waves related to cardiac pulsation and respiration [14–16]. The tethering in the spinal cord is via the denticulate ligaments between the pia mater and dura mater [17, 18]. Cardiac pulsation often triggers longitudinal pulsatile motions in the spinal cord and in connection with cerebrospinal fluid (CSF) flow; the spinal cord also experiences small fluctuations in the area [14, 19–21]. Spinal cord motion comprises of the direction, magnitude of total displacement, and velocity of motion [14, 22, 23]. The CSF flow is often correlating with cycles of cranial as well as caudal cord motion in a normal spinal cord [14, 24–26]. Normally, nerve roots do not come under tension during physiological motion in an intact spinal cord and thus do not exhibit any pain symptoms until during an injury [14]. Therefore, spinal cord motion may be reduced at the injury site due to subarachnoid scarring [14].

SCI characteristically has an injury epicenter where there is emergent tissue necrosis as well as cavity formation, axonal demyelination, glial stimulation, axotomy and scarring, and analogous endogenous repair activities such as neoangiogenesis and axonal sprouting [14, 27]. Cord swelling, inflammation, and tissue softening with areas of necrosis which ultimately become a cavity are the pathological processes during the acute and subacute periods after a severe SCI [14, 28, 29]. Acute SCI often results in vascular changes with loss of neurons, oligodendroglia, and astrocytes [3]. Also, neuroinflammation occurs with resultant invasion of the injury by a variability of inflammatory cells. Inflammatory cascades such as neutrophils, macrophages/-microglia, and T-cells, as well as humoral components like cytokines, interleukins, interferons, and prostaglandins, are often triggered during SCI [3]. Apart from the triggering of inflammatory cascades, the acute phase also involves hemorrhage, ischemia, excitotoxicity, and oxidative stress resulting in secondary cell death and degeneration of more tissue [3, 30].

The acute phase is associated to Wallerian degeneration of ascending and descending tracts with gradual formation of cavities in the cord, and the formation of the glial scar decreases significantly the growth capabilities of axons across the injury [31–33]. The blockade of nerve conduction results in paralysis as well as temporary loss of neural functions by spinal shock [3, 34, 35]. The associated oxidative stress results in the reduction of glutamate transport in astrocytes, thereby stimulating excitotoxicity because of augmented extracellular glutamate [3, 36]. The resultant ischemia triggers necrotic cell death in the epicenter of the injury. This process is usually the mechanism via which the induction of destructive signaling cascade occurs and expands to cause tissue damage [3, 37–39]. In response to edema, several vasoactive factors such as thromboxane, leukotrienes, platelet aggregation factors, serotonin and endogenous opioids are released [3, 40, 41]. This mechanism results in hypoperfusion, hypoxia, and hypoglycemia [3, 40, 41].

After ischemia follows a period of reperfusion which results in an increase in free reactive oxygen species (ROS) [3, 42, 43]. The generation and release of ROS are often the mechanisms via which the secondary injury process and the maintenance of a degenerative environment occur [3, 43]. On the other hand, complete loss of a grey matter with some preserved parenchyma, a margin of pia and fibroblastic scar, and an underlying thin rim of preserved gliotic white matter are the pathological cascade at the epicenter of most damaged spinal cords during the chronic period [3, 44–46]. An ideal treatment must be efficient in triggering axon regeneration in the injured central spinal cord and also attenuating scarring. It must also be capable of the generating growth-inhibitory factors at the lesion site as well as stimulating axon growth [32, 47, 48].

4. Neural Stem Cells

Neural stem cells (NSCs) (Figure 1) have been obtained from various regions of the brain from mice, rats, monkeys, and humans [3, 49]. Fetal NSCs and adult NSCs are the main types of NSCs. Fetal NSCs can be expanded for a long period *in vitro*, while adult NSCs have more partial abilities [49, 50]. Nevertheless, both cell types have an ineffective differentiation potential into neurons after numerous *in vitro* routes when they are transplanted into *in vivo* models [51, 52]. Several studies have demonstrated that adult NSCs are located in the spinal cord and usually around the central canal with narrow extension to the ventricular system stretching across the length of the spinal cord [53–56]. Analogous clones were capable of proliferating from both medial and lateral parts of the spinal cord [57, 58]. Nevertheless, minor multipotencies with few passages were observed during the later parts of the coning process [57, 58].

NSC proliferation in the spinal cord differs from that of the NSCs from the forebrain, where neurogenesis was observed to be sustained throughout the organism life [58]. NSCs from the spinal cord also require a distinctive mitogen *in vitro*, fibroblast growth factor-2 (FGF2) (Table 1) instead of epidermal growth factor (EGF) utilized for NSCs from the brain [55]. Human NSCs were cultured as neurospheres survived, migrated, and secreted differentiation markers for neurons and oligodendrocytes (Table 1) after long-term transplantation in SCI [59, 60]. Transplantation embryonic NSCs in aged mice were capable of improving functional recovery after SCI via the reformation of the cord microenvironment by stimulating the local secretion of growth factors, particularly the hepatocyte growth factor (HGF) (Table 1) [61]. NSCs have demonstrated to be capable of secreting $CD133^{+}/CD\ 34^{-}/CD45^{-}$ [62] (Table 1). *In vitro* studies have shown that EGF and FGF2 are fundamental factors in the cell culture conditions that sustained cell division with NSC [63–66].

Perrin et al. transplanted lentiviral-transduced human fetal neural progenitor cells (NPCs) capable of secreting neurogenin-2 (Table 1) into adult rats and observed that functional recovery correlated with partial restoration of serotonin fiber density caudal to the lesion [67]. Studies has shown that transplanted human NPCs obtained from fetal CNS tissues stimulated regeneration of the host corticospinal tract in the spinal cord with motor functional improvement compared to NPCs with brain characteristics [68–71]. Nevertheless, the major disadvantage with NPCs is the risk for tumorigenicity due to the contamination of undifferentiated cells prior to transplantation [72, 73]. Thus, to prevent tumorigenicity, contaminated cells were eliminated using the γ-secretase inhibitor (GSI), which inhibits Notch signaling (Table 1) [72]. The status of undifferentiated NPCs is regulated via the Notch signaling, and the blockade of this signaling triggers further maturation as well as neuronal differentiation of NPCs [72].

5. Transplant Cells from Neural Origin

Schwann cells (SCs), Olfactory ensheathing cells (OECs), and ependymal cells are the main transplant cells from neural origin [3, 74–76]. SCs and OECs have a number of morphological as well as molecular markers but have distinctive embryonic origins [75, 76]. SCs are derived from the neural crest while OECs originate from the olfactory placode [3, 74]. It is noteworthy that both cell categories secrete p75, GFAP, S100, and cell adhesion molecules like L1 and N-CAM (Table 1) [3, 74]. Furthermore, both cell categories secrete extracellular molecules like fibronectin and laminin [74].

SCs (Figure 1) are the ancillary glial cells of the peripheral nervous system (PNS) which stimulates the formation of myelin sheaths around peripheral axons as well as associated with intimate axonal glial intercommunications that grant axonal maintenance and impulse conduction [3, 77]. SCs often differentiate as well as proliferate and express distinctive neurotrophic factors which offer natural assistance for axonal regeneration after peripheral nerve injury [3]. Neurotrophic factors (Table 1) such as the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF) are often expressed by transplanted SCs [78]. These factors are capable of remyelinating injured axons, chaperon-regenerating axons, and accelerate the invasion of host SCs into the injured spinal cord section [79–81].

Some studies have shown that SC transplantation alone resulted in enhanced recovery of locomotory function in rodents while others demonstrated contrary results [82–85]. Furthermore, other studies showed very little recovery when SCs were simultaneous with methylprednisolone, neurotrophins, IL-10 (Table 1), and OECs at the cord stumps [86–88]. In view of these conflicting studies, more studies are warranted in this direction to determine the actual therapeutic roles of SCs in SCI.

OECs (Figure 1) are a distinct category of the glia situated in the olfactory system [89, 90]. In the olfactory system, the renewal of sensory neurons occurs constantly throughout life [89, 90]. OECs are capable of migrating within the central nervous system (CNS) and coexist in an astrocyte-rich locations [90, 91]. Studies have shown that axons of the new neurons are sheathed by OECs that chaperon and assist in their elongation as they cross from the PNS of the olfactory mucosa to the CNS of the olfactory bulb, where axons make new synaptic connections with other neurons [92–94]. OECs also possess neuroprotective abilities such as the expressing of trophic factors, decreasing of astroglial reactivity, and intercommunicating with damaged axonal pathways [94, 95].

Transplanted OECs were capable of remyelinating as well as improving axonal conduction in the demyelinated pathways of the rat SCI models [77, 96]. The effects of transplanted OECs were observable after acute than delayed transplantation. However, current studies demonstrated that chronic transplants are still efficient in the improvement of recovery during SCI [97–99]. Nevertheless, some studies did not find any significant neuroprotective/regenerative role of OECs in SCI [82, 100–102]. In view of these conflicting studies, more studies are warranted in this direction to determine the actual therapeutic roles of OECs in SCI.

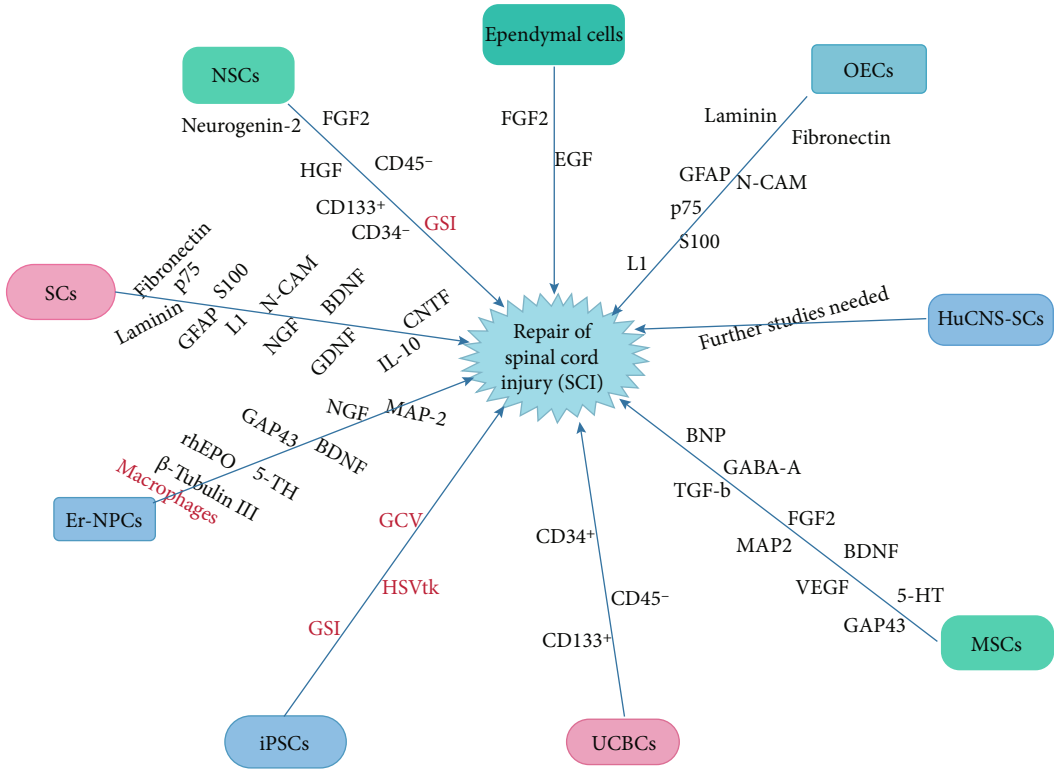

Figure 1: An illustration of the various types of stem cells and the pathways via which they influence the repair of spinal cord injury. Red: inhibitory pathway/tumor inhibition; black: facilitatory pathway; NSCs: neural stem cells; SCs: Schwann cells; OECs: olfactory ensheathing cells; Er-NPCs: erythropoietin-releasing neural precursors cells; UCBCs: umbilical cord blood cells; iPSCs: induced pluripotent stem cells; HuCNS-SCs: human central nervous system stem cells; MSCs: mesenchymal stem cells.

Ependymal cells (Figure 1) are the ciliated cells lining the central canal of the spinal cord. These cells propel the CSF as well as form a barrier to the spinal cord parenchyma [7]. Studies have demonstrated that transplanted ependymal cells self-renew in reaction to SCI as well as differentiated into oligodendrocytes and astrocytes [103–105]. Sabelström et al. demonstrated that blockade of ependymal cell proliferation after SCI rigorously impeded glial scar formation and resulted in augmented neuron loss [106]. Moreover, harvested and cultured ependymal cells were efficient in differentiating into astrocytes, oligodendrocytes, and neurons [106].

Kojima and Tator observed an upsurge in proliferation of ependymal cells as well as enhanced functional recovery when they fused growth factors EGF and FGF2 (Table 1) into the central canal after SCI [107]. Thus, they indicated that manipulation of ependymal cell could be a potential alternative to exogenous stem cell transplantation [107]. Other studies have also proven that ependymal cells are the endogenous stem cells in the adult spinal cord and thus form an alternative cell population to earmark for the treatment of SCI [56, 108].

Human central nervous system stem cells (HuCNS-SCs) (Figure 1 and Table 1) have also proven to be successful cells for transplantation after SCI [109]. They can be propagated, cryopreserved, and banked, while maintaining critical biological activity of self-renewal and engraftment, paracrine effects from expressed factors to improve neural plasticity, migration, and trilineage differentiation such as neurons, oligodendroctyes, and astrocytes [109].

6. Erythropoietin-Releasing Neural Precursor Cells

Erythropoietin-releasing neural precursors cells (Er-NPCs) (Figure 1) are very promising in the treatment of SCI [31]. These cells were initially referred as postmortem neural precursor cells [31, 110]. Initial studies demonstrated that intravenous infusion of Er-NPCs isolated from the subventricular zone (SVZ) six hours after the donor's death enhanced hind limb functional recovery but the cells were phagocytized by macrophages and the process of recovery stopped [31, 111, 112]. Er-NPCs accumulate at the lesion site and differentiate mainly into cholinergic neuron cells which were capable of protecting the myelin via the reduction of posttraumatic neuroinflammation [113–116].

Studies further demonstrated that Er-NPCs confine to the edges of the injury site where the microenvironment is often absolutely influenced by the prevailing neutralization of reactive inflammation [31, 113, 116]. Studies also showed that the neuroprotective role of transplanted Er-NPCs supports the structural maintenance or neoformation of an auspicious milieu [31]. This is exhibited by the higher maintenance of neuronal markers (Table 1) like β-tubulin III and MAP-2 at the lesion site [31]. Also, TH-positive fiber thickness in the ventral segment of the lumbosacral cord of

Table 1: The immune players influenced by the various stem cell types at the injury microenvironment after transplantation in SCI.

Type of cells	Immune players influenced at injury milieu	Effects on recovery	Citations
Neural stem cells (NSCs)	FGF2	Facilitatory	[55]
	HGF	Facilitatory	[61]
	$CD133^+/CD34^-/CD45^-$	Facilitatory	[62]
	Neurogenin-2	Facilitatory	[67]
	GSI	Tumor inhibition	[72]
Schwann cells (SCs)	p75	Facilitatory	[3, 74]
	GFAP	Facilitatory	[3, 74]
	S100	Facilitatory	[3, 74]
	L1	Facilitatory	[3, 74]
	N-CAM	Facilitatory	[3, 74]
	NGF	Facilitatory	[78–81]
	BDNF	Facilitatory	[78–81]
	GDNF	Facilitatory	[78–81]
	CNTF	Facilitatory	[78–81]
	IL-10	Facilitatory	[86–88]
	Fibronectin	Facilitatory	[74]
	Laminin	Facilitatory	[74]
Olfactory ensheathing cells (OECs)	p75	Facilitatory	[3, 74]
	GFAP	Facilitatory	[3, 74]
	S100	Facilitatory	[3, 74]
	L1	Facilitatory	[3, 74]
	N-CAM	Facilitatory	[3, 74]
	Fibronectin	Facilitatory	[74]
	Laminin	Facilitatory	[74]
Ependymal cells	EGF	Facilitatory	[107]
	FGF2	Facilitatory	[107]
Human central nervous system stem cell (HuCNS-SC)	Further studies needed	Facilitatory	[109]
Erythropoietin-releasing neural precursors cells (Er-NPCs)	Macrophages	Inhibitory	[31, 112]
	rhEPO	Facilitatory	[117]
	5-TH	Facilitatory	[31, 116, 117]
	GAP43	Facilitatory	[31, 118–120]
	BDNF	Facilitatory	[116, 122, 123]
	NGF	Facilitatory	[116, 122, 123]
	β-Tubulin III	Facilitatory	[31]
	MAP-2	Facilitatory	[31]
Mesenchymal stem cells (MSCs)	5-HT	Facilitatory	[137]
	GAP43	Facilitatory	[128, 147]
	BDNF	Facilitatory	[4, 148]
	VEGF	Facilitatory	[4, 148]
	FGF2	Facilitatory	[4, 148]
	MAP-2	Facilitatory	[4, 151]
	GABA-A	Facilitatory	[4, 151]
	BNP	Facilitatory	[4, 152, 153]
	TGF-β	Facilitatory	[4, 161, 162]
Induced pluripotent stem cells (iPSCs)	GSI	Tumor inhibition	[1]
	HSVtk	Tumor inhibition	[1]
	GCV	Tumor inhibition	[1]
Umbilical cord blood cells (UCBCs)	$CD34^+/CD45^-$	Facilitatory	[3]
	$CD133^+$	Facilitatory	[3]

injured Er-NPC-treated mice was much higher than that of saline-treated injured mice [31].

Cerri et al. demonstrated that exogenous administration of rhEPO (Table 1) augmented the protection of TH-positive (Table 1) fibers and sheaths the injured as well as its associated larger descending spinal and ascending cortical evoked potential [117]. This was accompanied by the substantial protection of parenchyma at the lesion site in the spinal cord as well as substantial attenuation of myelin loss in the ventral and medioventral pathways [117]. Furthermore, descending 5-HT and fibers containing catecholamine that distinctly reinnervate the caudal cord were also significantly boosted [116, 117]. Growth-associated protein-43 (GAP43) (Table 1) is a marker of axonal growth cones [31].

Studies have shown that GAP-43 secretion correlated with axon regrowth abilities and its higher secretion in the caudal cord sustained the improved regeneration across the lesion in Er-NPC-transplanted mice [118–120]. Furthermore, studies revealed that Er-NPCs were capable of enhancing the functional recovery as well as supported axon regeneration via the provision of an auspicious environment and the expression of EPO that had influential anti-inflammatory activity which were capable of decreasing the secretion of inflammatory cytokines which resulted in the neutralization of invasion via the inflammatory cells at the injury site [31, 33, 121].

Therefore, Er-NPCs have both anti-inflammatory and neuroprotective actions which lead to spinal tissue sparing as well as a positive microenvironment which allows for axonal regeneration across the injure site [31, 33, 121]. Compared to regular adult NSCs, transplanted Er-NPCs had a higher survival ability in a hostile environment. Studies have shown that Er-NPCs were capable of infiltration with inflammatory cells like macrophages and neutrophils, which influenced secondary degeneration [113, 116]. Also, the local production of growth factors like BDNF and NGF (Table 1) was augmented which resulted in the stimulation of neuronal survival as well as axonal regrowth [116, 122, 123].

7. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) (Figure 1) compose of cells that are self-renewing and have the ability to differentiate into various mesodermal tissues such as the bone, cartilage, muscle, and fat [4, 124–126]. Several studies have demonstrated that MSCs are capable of differentiating into neurons and glia, therefore favorable trophic agents as well as cell sparing agents [127–129]. They are capable of triggering several neurotrophic factors and cytokines as well as differentiating into several phenotypes [130]. Furthermore, they are capable of influencing inflammation as well as stimulating the generation of reparative growth factors [4, 131, 132]. MSCs were injected directly into the lesion site in most studies. Nevertheless, successful administration of MSCs via intrathecal, intravenous, or even lumbar puncture has also been reported [133–136].

Hofstetter et al. demonstrated that immature astrocytes obtained from bone marrow stromal cells and administered into the injured spinal cord were capable of stimulating the outgrowth of 5HT-positive fibers (Table 1) because they proposed growth-permissive surfaces [137]. Several studies have shown that the positive efficiency of stem cell transplants in the injured CNS was a result of the expression of trophic factors by the engrafted cells [138–140]. In most studies, the MSCs were transplanted during the acute or subacute phase of the injury with good results [138–141]. Nevertheless, in a few studies, the cells were transplanted at the chronic phase after spinal cord contusion with good results [142–144].

Some studies observed that acutely injected bone marrow MSCs stimulated more tissue sparing than delayed injected cells and their influence was observed as cell survival during the first week postinjection [145, 146]. Chopp et al. observed that intramedullary transplantation of MSCs one week after SCI enhanced functional outcome over a five-week period, with a few cells secreting neural markers [128]. Furthermore, studies observed substantial enhancement in neurological outcome at four months after transplantation with augmented GAP43 (Table 1) secretion among reactive astrocytes in the scar boundary as well as SVZs in rat SCI models [128, 147]. MSCs were also capable of augmenting astrocytic survival as well as increased astrocytic BDNF, vascular endothelial growth factor (VEGF), and FGF2 (Table 1) after ischemic injury *in vitro* [4, 148].

Studies have shown that MSCs were able to proliferate as well as migrate into the injured cortex and also secreted markers for both neurons and astrocytes [4, 149, 150]. Transplanted MSCs also secreted both neuronal marker MAP-2 and γ-aminobutyric acid A (GABA-A) receptors (Table 1) [4, 151]. Transplanted MSCs were capable of accelerating recovery in SCI via the expression of brain natriuretic peptide (BNP) (Table 1) as well as vasoactive factors which decreased edema and intracranial pressure as well as increased cerebral perfusion [4, 152, 153]. Also, mouse MSCs transplanted into the rat spinal cord moved towards the injury site within four weeks after transplant which was observed *in vivo* using fluorescence tracking with GFP [4, 150]. Furthermore, the migrated cells expressed neuronal or astrocytic markers [4, 150].

MSC-derived Schwann cells and Matrigel, a synthetic scaffold material, were capable of stimulating axonal regeneration as well as functional recovery after total transection of the adult rat spinal cord [4, 154, 155]. MSCs were also capable of triggering the electrophysiological features of neurons which means that MSCs have neuronal replacement potentials [156–158]. Furthermore, some studies observed cosecretion of markers from distinctive neural lineages in the same cells [156, 159, 160]. Transplanted MSCs were also able to trigger high levels of transforming growth factor-β (TGF-β) (Table 1) which was able to lessen the formation of scar tissue [4, 161, 162].

8. Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) (Figure 1) show features analogous to those of embryonic stem cells (ESCs) and are capable of generating all three germ layers [1, 73]. Thus, iPSCs are capable of improving ectodermal neural-lineage cells with suitable culture stimulation [1]. Human iPSC-NPCs were able to boost axonal regrowth,

angiogenesis, and maintenance of the whole spinal cord [1, 73]. Thus, iPSC-NPCs were able to influence neurological and electrophysiological recovery [1]. Nevertheless, the cellular features differ according to iPSC lines and some of the iPSC-derived NPCs generated tumors after being transplanted into CNS tissues [1]. Relatively, transplanted NPCs differentiated into three neuronal lineages without developing tumors [163]. Thus, insecure and unsteady human iPSC lines are capable of developing tumors after transplantation [163].

Nagoshi et al. observed that when tumorigenic human iPSC-NPCs were preserved with GSI (Table 1) for only one day *in vitro*, they showed neuronal differentiation, decrease in cell proliferation, and downregulation of tumor-related gene secretion [1]. When they grafted them in the SCI model of NOD/SCID mice, the iPSC-NPCs primarily produced mature neurons around the injury site without tumor formation for about 89 days after the grafting [1]. Comparatively, non-GSI-treated NPCs developed tumors as well as regression of motor function [1]. They concluded that pretreatment with GSI was capable of eradicating tumor-stimulating cells in human iPSC-NPCs [1].

Nagoshi et al. transduced the herpes simplex virus type I thymidine kinase (HSVtk) gene into tumorigenic human iPSC-NPCs and observed that HSVtk phosphorylates its prodrug ganciclovir (GCV) (Table 1) resulting in the generation of cytotoxic GCV phosphate which eliminated immature and/or proliferating tumor cells whilst sparing postmitotic mature neural cells [1]. They observed preservation of matured neuronal cells as well as boosted locomotor function when they grafted the iPSC-NPCs transduced with HSVtk into a rodent SCI model [1]. Thus, it indicated that only the tumorigenic cells were ablated after GCV injection [1]. The HSVtk/GCV system was adopted in clinical trials without any safety problems [164, 165].

Several studies demonstrated the effectiveness of iPSC-NPC grafting in chronic SCI notwithstanding the associated complications [1, 166]. Okano et al. elucidated the potential beneficial effect of iPS-derived NS/PCs for the repair of SCI and observed that careful preassessment of each iPSC clone prior to any clinical trial of human CNS illnesses was essential [167]. Uezono et al. described the efficacy of pretreatment against neural inflammation linked with iPSC-NPC grafting [168]. They observed that after iPSC-NPCs were grafted in this reformed milieu of SCI, the cells triggered copious synaptic connections with host neurons, which stimulated functional locomotor recovery [168].

9. Umbilical Cord Blood Cells

Umbilical cord blood cells (UCBCs) (Figure 1) have demonstrated to have therapeutic potential in various areas of medicine [169]. UCBCs are relatively easy to collect and possess distinctive features which make these cells exceptionally well customized for use as cellular treatments. UCBCs have a high rate of hematopoietic stem and progenitor cells; they are native of the immune cells and also possess nonhematopoietic cells that have therapeutic potentials [169]. Initial study demonstrated that human cord blood leukocytes were advantageous in reversing the behavioral effects of SCI, even when transplanted five days after injury [170, 171].

Also, human UCBCs transplanted into injured rat spinal cord models revealed that the UCBCs appeared in injured areas, but not in noninjured areas of rat spinal cords [170, 171]. Furthermore, the cells were never detected in analogous areas of the spinal cord of noninjured animals. These findings were coherent with the postulation that UCBCs migrate to and partake in the healing of neurological defects after SCI [170, 171]. Studies further demonstrated that transplanted human UCBCs differentiated into several neural cells, stimulated renewal of spinal cord tissue, and enhanced motor function in SCI rat models [172–175].

Moreover, molecular and ultrastructural analyses demonstrated that human UCBCs were capable of augmenting neuronal and oligodendrocyte survival in the injured zones [175, 176]. Cho et al. observed recovery of somatosensory evoked potentials as well as phenotypic differentiation of transplanted human UCBCS into oligodendrocytes [177]. Enormous quantities of non-ESCs are available in UCB comprising of a mixture of distinctive types of stem/progenitor cells, hematopoietic cells such as HSCs and $CD34^+/CD45^-$, and endothelial cells like $CD133^+$ stem cells (Table 1) [3].

10. Methods of Transplantation of Stem Cells in SCI

Currently, local and intravascular approaches are the main methods of administering cellular therapeutics to the spinal cord [178]. Local administration is often attained via either direct cellular injection into the spinal cord parenchyma or intrathecal administration into the subarachnoid space [178]. However, intravascular approaches often include both intra-arterial as well as intravenous routes [178]. The intraparenchymal route demonstrated the greatest transplantation efficiency with several differentiated cells within the injured parenchyma [178]. Nevertheless, with the intrathecal route, few transplanted cells were detected on the surface of the lesion site as well as on other areas of the uninjured spinal cord and the transplanted cells differentiated into neurons, astrocytes, and oligodendrocytes [178].

In both routes above, cells which were transplanted cells were not detected at off-target sites or outside of the spinal cord [178]. In most studies that the transplanted cells were administered intravenously, cells did not migrate to the injury site in the spinal cord but rather migrated to the lung, spleen, and kidney [178]. Also, many mice in the intravenous group died soon after transplantation as a result of possible pulmonary embolism [178]. Furthermore, the study observed a similar pattern of graft survival, with signal loss or cell death happening in the first week after transplantation on longitudinal bioluminescence imaging [178].

Moreover, in a mouse contusive SCI model, neural stem/-progenitor cell transplantation was compared between intralesional and intrathecal and intravenous administrations [109, 179]. Transplanted cells were highest in the intralesional hand-held injection group, and after approximately six weeks posttransplantation, cell luminescence declines to about 10% of their original level at the site of injury [109,

179]. In the intrathecal group, transplanted cell luminescence was scattered all over the subarachnoid space soon after transplantation [109, 180]. It was detected at the injury site pial surface one week later, and by six weeks, it had decreased to about 0.3% of the initial level [109, 180]. In the intravenous group, no grafted luminescent cells were found at the injury site but all of these mice exhibited cell accumulation in the chest, signifying pulmonary embolism [180].

11. Factors Influencing Spinal Injection Techniques and Challenges

Understanding the spinal cord structure as well as tissue properties, motion, blood supply, injury responses, and the properties of injection devices may assist in understanding the problems associated with therapeutic injections in SCI [14]. It is challenging to understand the importance of volume in spinal tissue. Nevertheless, a single nanoliter (μl) occupies a sphere with a radius of 124 μm, which is a significant space within spinal cord tissue [181]. A study revealed that the absolute volume of the human T8 spinal cord segment is 690 mm^3 or 690 μl [14, 181]. Some authors have demonstrated that the lesion volume in the spinal cord will be about 350 μl using a theoretical dimensions of 3.5 mm radius × 18 mm length as a model cylinder of the T8 spinal cord segment and permit of 1 mm of the conserved tissue rim [181]. The same idea above can be applied to other segments of the spinal cord during injection of stem cells.

Fluid clearance is a possible means of augmenting compliance during spinal injections [14, 182]. A study in the brain revealed that some fluid clearance may occur via bulk fluid flow as well as diffusion along the extracellular spaces and absorption to the CSF or blood particularly via the white matter [14, 182]. It is proven that the extracellular spaces are open increasing this flow during focal edema in the CNS. Nevertheless, these extracellular channels comprising of membrane interstices as well as ground substance may be dilated after trauma resulting in acutely generated extreme pressures [14]. The rates of brain fluid clearance have usually been measured in hours and not minutes [14]. A study demonstrated that with very slow pressure injections of solutes, a diffusion rate of 44 μl/5 min correlated with exceptional tissue conservation [14, 183]. Thus, at the above rate, it would take 114 min to administer 1 μl [14].

Studies have shown that the fundamental forces generated within the spinal cord tissue during injection correlate with tissue's properties like compliance, elastic modulus, stress, and strain, as well as susceptibility to radial tensile stress—tearing stress—as a result of pressure gradients [14, 184, 185]. The injection of stem cells in animal spinal cord models usually involves the immobilization with frames, micromanipulators, syringes, and syringe pumps like those manufactured by Hamilton, Kopf, Stoelting, Harvard, and other companies [14].

On the other hand, human injection of stem cells involves medically approved devices, specifically those which are disposable [14]. This makes it more difficult to use innovative solutions during experimental injections. Thus, for human injections, factors such as practicability of usage in the operating room, quick assembly, reliability, potential for sterilization, reproducibility, nonobstruction of the visual field by the surgical microscope, and resistance to accidental perturbation or disarticulation once deployed within the spinal cord are often considered [14]. They are two types of injection techniques described in human studies [14].

The first ones are the hand-held syringe injection techniques, which are founded on the interoperator changeability based on the depth of placement, motion during injection, and injection rates. These techniques are simple, more rapid to deploy, and more flexible based on the approach angles [14, 186]. The second type is the use of a surgical operating table-mounted stereotaxic device. In this technique, enormous quantities of small injections were delivered into the damaged cord as well as the normal cord above and below the injured site [14, 187]. This device-stabilized technique of administering cells offers the capacity to accurately target single or multiple sites within the spinal cord just like in cranial stereotactic techniques [14, 187].

Preclinical grafting of cell in rodent studies has essentially utilized the stereotactic frame modified as a syringe and needle holder or free-hand injections [4, 180, 188]. The hand-held syringe injection technique often permits the surgeon to compensate for negligeable systolic and/or respiratory movements [109]. Clinically, syringe positioning device injections in subacute to chronic SCI have also been investigated [109]. Table-mounted syringe positioning devices or patient-anchored, retractor-based, syringe positioning devices which are often rigid or attached to a floating cannula are the two options for syringe stabilization [109]. The quantity of tissue dissection needed to anchor the device which could result in spinal instability like kyphosis is the possible shortcoming of the retractor-mounted or patient-anchored devices [109].

Several clinical trials utilized hand-held cellular injections after acute, subacute, and chronic SCI [109, 189]. The cell types that were used in these clinical trials included bone marrow MSC, activated macrophages, and autologous OECs [189–192]. In another study, the C4-T6 segment of the spinal cord was trimmed of scar tissues subsequent to the transplantation of OECs in the defect [189]. These authors observed improvement in motor function in the transplanted subjects [189]. Furthermore, long-term follow-up in at least one subject revealed the occurrence of a mass comprising of mucoid cysts with respiratory epithelium origin eight years post-transplant [191, 193]. In other studies, some cell types subsequently displayed substantial migration while others do not. SCs or bone marrow MSC displayed the ability to extemporaneously form linear bundles parallel to white matter tracts [14, 129].

12. Cell Transplantation and Spinal Cord Repair

As soon as SCI occurs, astrocytes proliferate as well as consolidate around the edges of the injury site to separate the damaged area from the surrounding healthy tissue [7]. Several studies have demonstrated that in the subacute phase, usually from one to two weeks after injury, reactive astrocytes migrate to the epicenter of the injury site and subdue

inflammatory cells resulting in tissue repair as well as functional improvement [194–196]. Studies have further demonstrated that the extended reactive astrocytes around the injured perimeter triggers a fibroblast-like pericyte resulting in the formation of the astrocytic scar, the key inhibitor of CNS axonal regeneration during the later phase of the SCI [197–199].

Contrarily, some studies have demonstrated that the glial scar was obligatory to inhibit the spread of injury as well as essentially boost CNS repair [200, 201]. Fibrotic scarring was initially described to originate from meningeal cells after CNS injury. Nevertheless, studies demonstrated that PDGFRβ-positive pericytes as well as CD13-positive endothelial cells are active sources of the cellular configuration of the fibrotic scar in SCI [202, 203]. Another study revealed that microvascular endothelial cells engulfed myelin debris via autophagy-lysosome pathway-triggered inflammation, angiogenesis, and fibrotic scar formation [204, 205].

After successful transplantation, implanted neural stem/progenitor cells (NSPCs) differentiate into neural cells that replace injured cells as well as offer local neurotrophic factors that aid neuroprotection, immunomodulation, axonal sprouting, axonal regeneration, and remyelination. Studies have shown that transplantation of NSPCs in the acute or subacute phases of SCI differentiate into oligodendrocytes, augmented the quantity of myelinated axons at the injure site, and boosted functional recovery [206–208]. Furthermore, studies showed that NSPC-derived myelin was fundamental in the remyelination process after SCI [208, 209]. Thus, this exhibits the significant role of remyelination in functional recovery triggered by stem cell transplantation approaches during SCI [209–211].

Lu et al. demonstrated that stem cells transplanted into the cavity and/or surrounding tissue regenerated as well as expressed neurotropic factors that triggered the growth of axons, both endogenous and graft-derived, across the lesion to form synapses as well as repaired spinal cord connectivity [212]. Studies have also shown that one of the mechanisms via which functional recovery happened in subjects with SCI was via neural plasticity or the capability of the CNS to regenerate its circuits over time [213–215]. Bonner et al. established that the neurons that differentiated from transplanted NSPCs prolonged axons as well as form new synapses with host neurons. They further indicated that the regenerated connections were mostly not exact reconnections of the lost neural circuits, but rather de novo circuits [216].

Assinck et al. demonstrated that oligodendrocyte precursor cells (OPCs) are primarily quiescent in the healthy CNS [217]. They indicated that OPCs are capable of proliferating and differentiating into mature oligodendrocytes in response to injury, which aid in remyelination [217] . Studies further showed that transplanted OPCs do not only complement the inadequate remyelination process of endogenous OPCs but also express neurotrophic factors that inhibited inflammation as well as promote axonal regeneration [218–220]. Studies have also proven that SCs myelinate peripheral nerve fibers and are capable of migrating into the injured spinal cord as well as boost remyelination after SCI [81, 188].

Several studies have demonstrated that transplanted SCs are capable of remyelinate axons as well as boost neural conduction just like OPCs [221, 222]. SCs are also capable of expressing growth factors, extracellular components, and adhesion molecules that triggered functional recovery after SCI [223–225]. Xu et al. demonstrated that NSCs transplanted into the lumbar ventral horn migrated to the central canal as well as triggered proliferation of ependymal cells and differentiated into neural precursors and neurons [226]. Salewski et al. established that ESC-derived NSPCs were capable of treating subjects with SCI without tumor formation [227].

Several studies have demonstrated that transplanted exogenous NSCs are capable of triggering neurogenesis in the spinal cord ependymal niche as well as boost the survival of the newly generated host neurons, which was analogous to the neurogenesis triggered in the brain SVZ via NSPC and MSC transplants [228, 229]. The key challenges associated with nerve repair are the lack of self-repair as well as neurotrophic factors, primary and secondary neuronal apoptosis, and factors that prevent the regeneration of axons locally (Figure 2) [230–232]. It was established that neurons that survive the initial traumatic damage may be lost due to pathogenic activities like neuroinflammation and apoptosis [232, 233].

Rong et al. demonstrated that NSC-derived small extracellular vesicles (sEVs) were capable of inhibiting neuronal apoptosis, microglia stimulation, and neuroinflammation resulting in the stimulation of functional recovery in SCI model rats [233]. They stressed that these outcomes above transpire as a result of neuronal autophagy [233]. SEVs are small vesicles expressed by cells that partake in cell-cell signaling via the transmitting of RNA, proteins, and bioactive lipids [234–236]. Studies have shown that the source of these vesicle correlated well with the expression of specific surface antigens [235–237]. Furthermore, several studies have demonstrated that sEVs generated by NSCs had therapeutic efficiency against ischemic, inflammatory, and neurodegenerative diseases [238, 239].

NSC-sEV was capable of inhibiting glutamate excitotoxicity *in vitro* as well as secondary SCI *in vivo* during pretreatment experiments (Figure 2) [233]. Also, NSC-sEV was capable of inhibiting neuroinflammation processes like microglial activation, nitric oxide (NO) expression, and cytokine production thereby stimulating autophagy (Figure 2) [233]. Thus, the antiapoptotic and anti-inflammatory actions of NSC-sEVs were directly dependent on the stimulation of autophagy (Figure 2) [233]. Several studies have demonstrated that although the pathogenic mechanisms of SCI are complex, inflammation and apoptosis are the two key processes that occur at the secondary phase of the injury [240, 241]. Several studies have demonstrated that after SCI, proapoptotic proteins Bax and cleaved caspase-3 are often elevated, while antiapoptotic Bcl-2 is normally decreased (Figure 2) [242, 243].

Furthermore, inflammation (Figure 2) involves triggering of microglia as well as elevation of neuroinflammatory cytokines like TNF-α, IL-1β, and IL-6 after traumatic SCI [240, 244].

Rong et al. demonstrated that LPS-induced NO generation by isolated microglia was downregulated by preincubation with NSC-sEVs [233]. Also, the secretory levels of

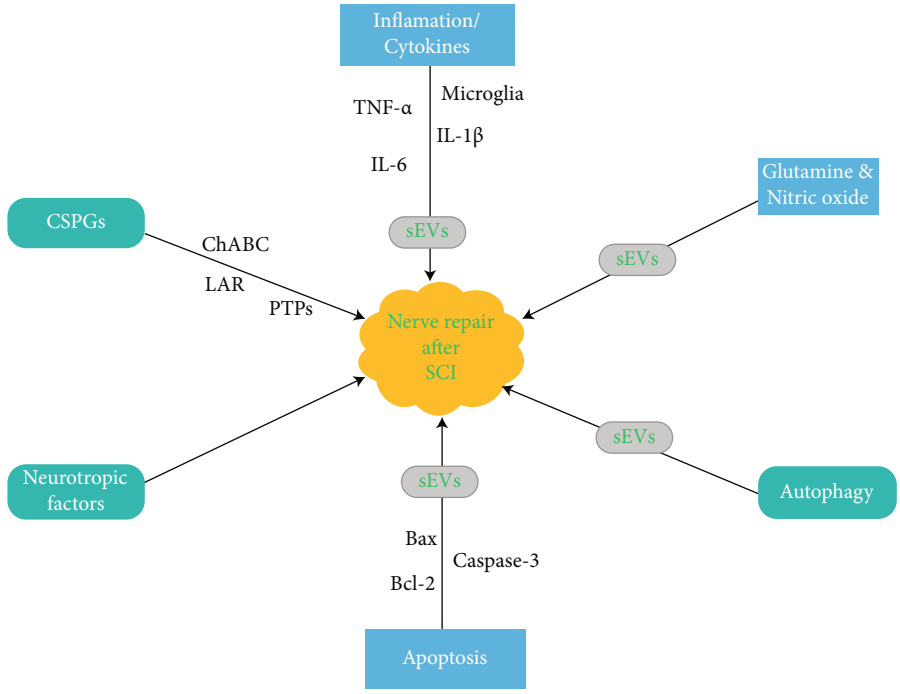

Figure 2: An illustration showing the nerve repair mechanisms after SCI. Nerve's lacking of self-repair. CSPGs: chondroitin sulfate proteoglycans; ChABC: chondroitinase ABC; LAR: leukocyte common antigen related; PTPσ: protein tyrosine phosphatase σ; sEVs: small extracellular vesicles.

proinflammatory cytokines were appreciably inhibited by NSC-sEV (Figure 2) pretreatment [233]. Furthermore, the quantity of activated CD68-positive microglia (Figure 2) was appreciably reduced in SCI pretreated with NSC-sEVs compared to the untreated injured spinal cord, signifying that NSC-sEV was capable of downregulating neuroinflammation *in vivo* as well as *in vitro [233]*.

Bradbury et al. demonstrated that mortification of chondroitin sulfate proteoglycans (CSPGs) by chondroitinase ABC (ChABC) was capable of breaking down the inhibitive barrier as well as stimulated endogenous pathological repair resulting in synapse reorganization as well as functional recovery in SCI subjects (Figure 2) [245]. Studies have demonstrated that a combination of stem cells and ChABC promoted functional recovery even in the chronic phase of SCI [246–248]. CSPG inhibition was facilitated by two members of the leukocyte common antigen-related (LAR) phosphatase subfamily and protein tyrosine phosphatase σ (PTPσ) [246–248]. Also, the LAR and PTPσ receptors were facilitated via the stimulation of oligodendrocyte differentiation and apoptosis by CSPGs in SCI [247, 249, 250].

13. Conclusions

Implanted stem cells are capable of differentiating into neural cells that replace injured cells as well as offer local neurotrophic factors that aid neuroprotection, immunomodulation, axonal sprouting, axonal regeneration, and remyelination. At the microenvironment of SCI, stem cells are capable of producing growth factors like BDNF and NGF which trigger neuronal survival as well as axonal regrowth. Although stem cells have proven to be of therapeutic value in SCI, the major disadvantage of some of the cell types is the risk for tumorigenicity due to the contamination of undifferentiated cells prior to transplantation. Local administration of stem cells via either direct cellular injection into the spinal cord parenchyma or via intrathecal administration into the subarachnoid space is currently the best transplantation modality for stem cells during SCI.

Abbreviations

GABA-A: γ-Aminobutyric acid
GSI: γ-Secretase inhibitor
BDNF: Brain-derived neurotrophic factor
BNP: Brain natriuretic peptide
CSF: Cerebrospinal fluid
CNTF: Ciliary neurotrophic factor
CNS: Central nervous system
CSPGs: Chondroitin sulfate proteoglycans
ChABC: Chondroitinase ABC
EGF: Epidermal growth factor
Er-NPCs: Erythropoietin-releasing adult neural precursors cells
ESCs: Embryonic stem cells
FGF2: Fibroblast growth factor-2
GCV: Ganciclovir
GDNF: Glial cell-derived neurotrophic factor
GAP43: Growth-associated protein-43
HuCNS-SC: Human central nervous system stem cell
HSVtk: Herpes simplex virus type I thymidine kinase
iPSCs: Induced pluripotent stem cells

LAR: Leukocyte common antigen related
MSCs: Mesenchymal stem cells
NSCs: Neural stem cells
NGF: Nerve growth factor
NSPCs: Neural stem/progenitor cells
NO: Nitroxide
OECs: Olfactory ensheathing cells
OPCs: Oligodendrocyte precursor cells
PNS: Peripheral nervous system
PTPσ: Protein tyrosine phosphatase σ
ROS: Reactive oxygen species
SCI: Spinal cord injury
SCs: Schwann cells
SVZ: Subventricular zone
sEVs: Small extracellular vesicles
TGF-β: Transforming growth factor-β
UCBCs: Umbilical cord blood cells
VEGF: Vascular endothelial growth factor.

Data Availability

No data was used for this paper.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors contributed toward literature search, drafting, and critical revision of the paper and agree to be accountable for all aspects of the work.

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