Stem Cells International

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Review Article

Application of Stem Cell Therapy for ACL Graft Regeneration

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Graft regeneration after anterior cruciate ligament (ACL) reconstruction surgery is a complex three-stage process, which usually takes a long duration and often results in fibrous scar tissue formation that exerts a detrimental impact on the patients' prognosis. Hence, as a regeneration technique, stem cell transplantation has attracted increasing attention. Several different stem cell types have been utilized in animal experiments, and almost all of these have shown good capacity in improving tendon-bone regeneration. Various differentiation inducers have been widely applied together with stem cells to enhance specific lineage differentiation, such as recombinant gene transfection, growth factors, and biomaterials. Among the various different types of stem cells, bone marrow-derived mesenchymal stem cells (BMSCs) have been investigated the most, while ligament stem progenitor cells (LDSCs) have demonstrated the best potential in generating tendon/ligament lineage cells. In the clinic, 4 relevant completed trials have been reported, but only one trial with BMSCs showed improved outcomes, while 5 relevant trials are still in progress. This review describes the process of ACL graft regeneration after implantation and summarizes the current application of stem cells from bench to bedside, as well as discusses future perspectives in this field.

1. Introduction

Anterior cruciate ligament (ACL) injuries account for more than 50% of all knee injuries [1], which may cause knee instability, resulting in meniscal damage and osteoarthritis. When tears occur, ACL reconstruction (ACLR) surgery is usually undertaken, which yields the best therapeutic effects and postoperative evaluation scores for patients [2]. There are over 175,000 patients undergoing ACL surgery annually in the US alone [3], but more than 10% of patients experience reinjuries, muscular atrophy, delay in healing, poor proprioception, and graft failure after reconstruction in long-term follow-ups [4–7]. Hence, the major challenge is how to improve postoperative graft healing.

After transplantation, the graft goes through a complex three-staged healing process involving necrosis, remodeling, and ligamentization, which may take around 2 years [8]. Moreover, fibrous scar tissue is often formed at the interface, instead of a natural insertion [9, 10]. When a tissue is characterized by poor healing capacity, such as tendon and ligament, regenerative strategies are usually considered. Several common regenerative approaches, such as stem cells, biomaterials, and bioactive molecules, have been investigated and proven to be effective [11–13]. Among these, stem cells are

extremely appealing, due to their self-renewal capacity, long-term viability, and multilineage differentiation potential [14]. In particular, mesenchymal stem cell (MSC) can differentiate into various terminally differentiated lineages, which can be utilized to engineer mesenchymal-derived tissues, and also promote healing by secreting various immunoregulatory molecules, such as paracrine trophic mediators [15, 16]. To induce stem cells to differentiate into a specific lineage, various differentiation inducers are usually utilized, such as recombinant gene transfection, growth factors, and biomaterials. Indeed, an increasing number of preclinical research studies have confirmed that inducers could enhance bone-to-tendon healing with better biomechanical properties and more mature tissue formation. Several clinical trials have been attempted, but so far, it is still uncertain whether stem cell augmentation could facilitate the healing process.

The purpose of this review is to describe the natural healing process after ACL graft implantation and summarize the current application of stem cells from bench to bedside, as well as discuss future prospects in this field.

2. Process of ACL Graft Regeneration

From the posterior part of the inner surface of the lateral femoral condyle, the ACL runs anteriorly, medially, and distally to the tibia [17]. The main component of ACL tissue is constituted of parallel and closely arranged collagen fibers, and fibroblasts are distributed along the long axial among the collagen fibers [18]. There are three characteristic stages of graft healing after ACL reconstruction in both humans and animals [19]: (i) early phase associated with necrosis and hypocellularity, (ii) remodeling phase associated with revascularization and cell activities, and (iii) ligamentization phase associated with restructuring towards the native ACL [20].

During the early stage, necrosis occurs in the graft centra, which leads to a release of various cytokines, such as tumor necrosis factor- (TNF-) α, interleukin (IL) 1-β, IL-6, and chemokines, which may trigger growth factor expression [21, 22]. Some host cells (neutrophils, macrophages, and MSC) migrate to the graft periphery [11, 12, 21], and towards the inner tendon [11]. Collagen fibrils begin disintegrating [13], and no graft revascularization could be observed [23, 24]. The collagen fibers of tendon display a bimodal distribution, with large collagen fibers constituting the majority. However, during healing, small fibers increase while large ones decrease (Figure 1(a)). Additionally, new surgery with attached graft may skip early necrosis, which retains the native blood supply [25, 26].

During the remodeling stage, large amounts of growth factors are released, which stimulates cell migration and proliferation as well as extracellular matrix synthesis and revascularization [22, 27, 28]. The hypercellular region at the perimeter consists of mesenchymal stem cells and fibroblasts [29]. Activated fibroblasts secrete various growth factors, which almost completely cease at the end of the remodeling stage [22]. The large diameter collagen fibrils get depleted [20], while the Sharpey-like fibers form to counteract shear stress and to attach the tendon graft to bone [30] (Figure 1(b)).

During the maturation stage, cellularity and mechanical properties become gradually similar to intact ACL but never reach the original levels [31, 32]. Progressive mineralization occurs, with subsequent bony ingrowth into the graft surface. Small collagen fibers predominate while large ones could hardly be seen, which differ significantly from normal ACL, with an unclear bimodal distribution (Figures 1(c) and 1(d)). Moreover, during this stage, more osteoarthritic changes and cartilage damage could be observed, with no significant differences in the expression of inflammatory cytokines or biomarkers [33].

Based on the above description, the graft healing process is slow and requires a long duration. The remodeling stage is finished by 9 months at the earliest [20, 34], and ligamentization could be observed after 2 years [8]. In the clinic, patients are usually recommended to return to low and moderate intensity exercise after 6 months [35–37], and typically regain about 85% function eventually [38]. Hence, a safe and effective approach to expedite the healing process is needed to restore the natural biomechanics of tendon, which is required for rapid return to preinjury activity levels.

3. Stem Cell Therapy for Graft Regeneration

Stem cells show remarkable ability for self-renewal, long-term viability, and multilinear culture [14], which is an essential element in tissue engineering technology. In different cultures, stem cells could differentiate into nerve cells, hepatocytes, or blood cells. Combined with materials science, it is possible to construct similar tissues and organs to substitute the injured part. It has been widely proven that stem cells are effective in many diseases, such as central nervous system damage, and corneal destruction [39, 40]. Recent scientific literature has demonstrated promising outcomes of stem cell augmentation for ligament reconstruction in animal models [41–43] (Figure 2 and Table 1). However, the application of stem cells in ACLR requires further consideration of cell resource, differentiation induction, and cell fate.

3.1. Selection of Stem Cell Sources. There are several common cell sources in tissue engineering, such as embryonic stem cells (ESCs), induced pluripotent stem cell (iPSC), adipose tissue-derived stem cells (ADSCs), bone marrow-derived mesenchymal stem cells (BMSCs), and tendon/ligament stem/progenitor cells (TDSCs/LDSCs). In particular, MSC is the focus of much interest, as these cells are easily isolated from a variety of adult tissues and cultured *in vitro*. Cells from different sources have varying propensities to differentiate into various tendon/ligament lineages, and hence, it is imperative to weigh the pros and cons of various different stem cell types (Table 2).

3.1.1. BMSC. BMSCs have multipotential capacity to differentiate into osteoblasts, chondrocytes, and adipocytes and hence have been most widely studied for enhancing tendon-bone healing, yielding satisfactory outcomes (Figure 2(d)). Sakaguchi compared the proliferative capacities of different stem cell types and observed that BMSCs were retained even at passage 10, whereas that of ADSCs was lost at passage 7 [44], thus

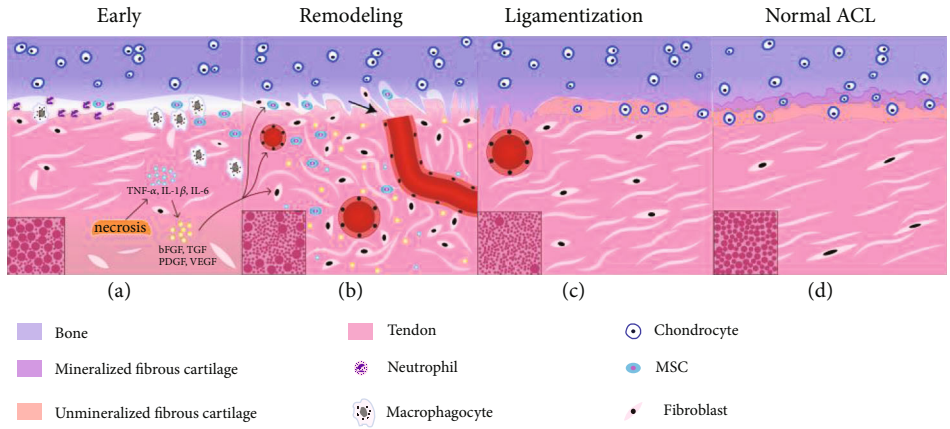

Figure 1: Schematic model for graft regeneration after ACL reconstruction. (a) Early stage characterized by necrosis, fiber disintegration, and cytokine release; neutrophils, macrophagocytes, and mesenchymal stem cells (MSC) can be observed in the interface in order, and then macrophagocytes and MSC migrate into the inner tendon. The collagen fibers displayed a bimodal distribution, with large ones constituting the majority; (b) remodeling stage marked with Sharpey fibers (arrow), cell migration, vascularization, ECM remodeling, various growth factor activities, and disordered organization of collagen fibers (bimodal distribution with small ones constituting the majority); (c) ligamentization stage marked with vascularization gradually disappearing, fibrocartilage formation, and ordered collagen with almost unimodal small fibers; (d) normal ACL, 4-layer direct insertion including ligament, fibrocartilage, mineralized fibrocartilage, and bone in order. The collagen fibers showed unclear bimodal distribution.

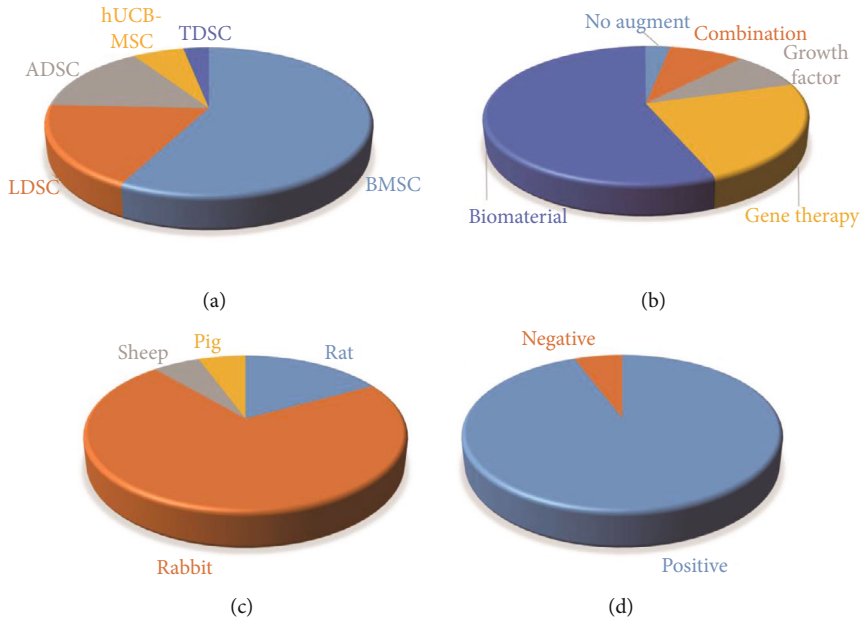

Figure 2: Features of included animal studies. (a) Cell resources; (b) augmentations; (c) animal models; (d) general study outcomes. BMSC: bone marrow-derived mesenchymal stem cells; ADSC: adipose-derived stem cells; hUCB-MSC: human umbilical cord blood-derived mesenchymal stem cells; TDSC: tendon-derived stem cells; LDSC: ligament-derived stem cells.

showing the greater stability of BMSCs. However, these cells are not considered as the optimal choice due to the risk of ectopic ossification and donor injury. The therapeutic effects of BMSCs are thought to result from migration of the cells to inflammatory sites and suppression of inflammation. They are rarely involved in colonizing the healing tissue as part of the tissue repair mechanisms [45].

Lim et al. [46] implanted hamstring tendon autografts into the bone tunnel in rabbits, which was coated with MSCs embedded within a fibrin glue carrier in one limb, and fibrin glue only in the other limb, resulting in cartilage-like insertions rather than scar tissue. A similar study showed that BMSCs could decrease tunnel widening [47].

3.1.2. ADSC. ADSCs have the advantages of abundant and ready availability, as well as capacity for secreting various factors, such as VEGF, hematopoietic factors, and immunoregulatory factors, to promote tissue repair and growth. Over 500 times more stem cells can be obtained from adipose tissue than from an equal tissue volume of bone marrow

Table 1: Recent animal studies on stem cell therapy for ACL graft regeneration.

	Author	Augmentation/induction	Animal	Evaluation	Outcome	Other outcomes
BMSCs	Hur	Fibrin glue	Rabbit	His, CT	+	
	Lim	Fibrin glue	Rabbit	His, Mech	+	
	Fan	Silk scaffold	Rabbit	His, Mech, CT	+	
	Fan	Silk scaffold	Pig	His, Mech, CT	+	
	Li	Triphasic silk graft	Rabbit	His, Mech, CT	+	
	Zhu	Electrospun scaffolds	Rabbit	His, Mech, CT	+	Lattice-like nanofibrous meshes enhance osteogenic differentiation
	Vaquette	PCL electrospun mesh	Sheep	His, Mech	+	
	Zhang	PLGA silk scaffold	Rabbit	His, Mech	+	
	Li	Cu-BG/PET	Rat	His, Mech, CT	+	
	Lu	Decellularized allogenic ST	Rabbit	His, Mech, CT	+	Decellularized allograft+BMSCs are better than allograft
	Setiawati	VEGF	Rabbit	His, Mech, MRI	+	
	Teng	PRP	Rabbit	His, Mech, CT	+	PRP enhances osteogenic differentiation
	Zhu	BMP2 gene therapy	Rabbit	His, Mech	+	
	Chen	bFGF/BMP2 gene therapy	Rabbit	His, Mech, CT	+	Combined BMP2 and bFGF exerted more potent effects than lone growth factor
	Wang	TGF gene therapy	Rabbit	His, Mech, CT	+	
	Dong	BMP2 gene therapy	Rabbit	His, Mech	+	
	Wei	TGFβ/VEGF gene therapy	Rabbit	His, Mech	+	Combined TGFβ-1 and VEGF165 exerted more potent effects than lone growth factor
	Li	PDGF gene therapy	Rabbit	His	+	
	Fan	Triphasic silk scaffold (TGF-β3 and BMP2 gene therapy)	Rabbit	His, Mech	+	
	Pauly	CTGF-electrospun scaffolds	Rabbit	His, X-ray	+	
	Kosaka	Fibrin glue	Rabbit	His, Mech	+	
	Teuschl	Silk scaffold	Sheep	His, CT	(-)	
	Parry	PCLF+PET scaffold	Rabbit	His, Mech, CT	+	
ADSCs	Kouroupis	Leeds-Keio biomaterial; BMP-2/FGF-2	Pig	His, Mech	/	BMP-2/FGF-2 induced stem cells to differentiate towards bone and ligament at the ends and central part of the biomaterial scaffold
	Zhang	Runx2 gene therapy	Rabbit	His, Mech, CT	+	Runx2 enhances osteoblast differentiation and inhibits adipogenic differentiation
	Mifune	Injected	Rat	His, Mech, CT	+	
	Mifune	Cell sheet	Rat	His, Mech	+	Cell sheet is better than injection
LDSCs	Ruan	Silk-collagen sponge scaffold	Rabbit	His, X-ray	+	
	Hu	SDF-1 releasing collagen-silk	Rabbit	His, CT	+	
	Takayama	VEGF gene therapy	Rat	His, Mech	/	CD34+ LDSCs have positive effects; overexpression of VEGF impairs biomechanics
	Kawakami	BMP2 gene therapy	Rat	His, Mech	+	BMP2 enhances osteogenic differentiation
TDSCs	Lui	Cell sheet	Rat	His, Mech, CT	+	
sMSCs	Ju	Gel injection	Rat	His	+	
hUCB-MSCs	Jang	Fibrin glue	Rabbit	His, CT	+	
	Park	3D bio-printed scaffold	Rabbit	His, CT	+	

PRP: platelet-rich plasma; His: histology; Mech: mechanics; PCLF+PET: polycaprolactone fumarate scaffolds with polyethylene terephthalate; bFGF: basic fibroblast growth factor; BMP2: bone morphogenetic protein 2; TGF: transforming growth factor; VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor; ST: semitendinosis; PCL: polycaprolactone; BMSCs: bone marrow-derived mesenchymal stem cells; ADSCs, adipose-derived stem cells; sMSCs: synovial mesenchymal stem cells; hUCB-MSCs: human umbilical cord blood-derived mesenchymal stem cells; PLGA: lactic-co-glycolic acid; Cu-BG/PET: copper-containing bioactive glass polyethylene terephthalate; Runx2; PCLF+PET: polycaprolactone fumarate+polyethylene terephthalate sutures; SDF: stromal cell-derived factor 1; CTGF: connective tissue growth factor; bFGF: basic fibroblast growth factor.

Table 2: The advantages and disadvantages of commonly utilized stem cell types in ACL graft regeneration.

Formulation	Msc content	Advantages	Disadvantages
BMSCs	0.01-0.001% [142]	Great proliferation Low cost Low immunoreaction Easy to obtain	Low content Donor pain and infection Less homogeneous
ADSCs	~1% [143]	Abundant resource More homogeneous Factor secretion Less immunogenic than BMSCs	Enzymatic processing Low ligament differentiative potential [53]
TDSCs/LDSCs	3-4% [56]	Same derived resource Better epigenetic regulation [144] Cell-line maintainment [145]	Slow growth Low content
ESC	—	Indefinite self-renewal [146] Totipotency	Ethic issue Tumorigenicity [147] Immunogenicity [148]

BMSCs: bone marrow-derived mesenchymal stem cells; ADSCs: adipose tissue-derived stem cells; TDSCs/LDSCs: tendon/ligament stem/progenitor cells; ESCs: embryonic stem cells.

[48], and proteomic analysis of ASC secretome identified a total of 2416 distinct proteins [49]. In addition, ADSCs show lower risk of ectopic ossification, with less immunogenicity than BMSCs [50], causing less damage to the donor site, without the limitations associated with age-related decline of BMSCs. Indeed, ADSCs have demonstrated their suitability for various cell therapy applications including angiogenicity, osteogenicity, immunomodulation, and promotion of tissue remodeling [51, 52]. However, a study showed that ADSCs cannot continuously upregulate ligament-related markers with growth factors *in vitro*, as it exhibits a bias towards adipogenic differentiation [53].

It has been reported that ADSCs promote the early healing processes of tendon and bone in rabbits [43]. But Teuschl et al. [54] found that additional ADSCs did not result in any additional benefit for osteointegration, as compared with the silk scaffold group histologically, which showed ambiguous function.

3.1.3. TDSC/LDSC. It has been reported that tissue-specific stem cells may retain a residual "epigenetic memory" of their tissue of origin [55]. When back at their tissue of origin, they could adapt to the environment better, survive longer, and differentiate more easily. TDSCs were first isolated from human hamstring tendon in 2007 [56], while a later study showed the possibility of isolating TDSCs from very small fragments of tendon tissue [57]. These cells proliferated faster, exhibited higher clonogenicity and less immunogenicity, and had more multilineage differentiation potential than BMSCs [58, 59]. However, the purity of TDSC populations is highly debatable, as it displayed lower adipogenic and osteogenic capacities than ADSCs [60], and lower multilineage differentiation potential than LDSCs [61]. TDSC-related studies are rare but seem promising, exhibiting high tenogenic potential and maintaining high chondroosteogenic gene expression [59].

Originating from the ligament tissue [62], CD90+CD73+ LDSCs tend to differentiate into ligament-committed cells or chondrocytes, as compared with BMSCs [63, 64]. The application of LDSCs *in vivo* has yielded generally positive results, when combined with silk scaffold, cell sheet, and injection [65, 66]. In particular, CD34+ vascular cells from ligament tissue are considered as another type of adult stem cell and have proven efficacious in tendon-bone regeneration [66, 67]. As a promising cell source, ACL-derived iPSCs are also under study [68]. The common problem of both is that low cell numbers necessitate expansion, which may influence phenotypic maintenance. Still, TDSCs and LDSCs are considered the most promising cell types for ACL regeneration.

3.1.4. Other Stem Cell Types. hUCB-MSCs: hUCB-MSCs have the advantages of noninvasive isolation method, superior tropism, and high differentiation potential. Transplantation in rabbits enhanced bone-tendon healing effectively, without immune rejection [69, 70], while the application of human amniotic mesenchymal stem cells (hAMSCs) is still under research [71].

Synovium-derived MSCs (sMSCs): after injury, a local increase of MSCs was observed, and these MSCs were identified as sMSCs rather than BMSCs [72]. sMSCs can potentially promote collagen fiber production, which resembles Sharpey's fibers at the early stage.

ESC/iPSCs: ESCs could differentiate into any tissue or cell type, but therapeutic applications of these cells have been subjected to serious and prolonged legal/ethical discussion. On the other hand, iPSCs avoided ethical issues associated with ESC and also offered the possibility for autologous regeneration of any tissue. Cord and peripheral blood are attractive sources of reprogrammable cells for generating iPSCs [73, 74]. As a promising cell source, ACL-derived iPSCs are still under research [68]. But current outcomes of therapeutic applications in animal models seem controversial, with transplantation of ESCs into the knee joint of mice resulting in teratoma formation and subsequent destruction of the joint [75]. By contrast, composite grafts with iPSCs in pigs showed similar morphological and biochemical characteristics to normal ACL [76].

Exosome: no related research studies have been reported yet. However, the application of exosomes in tendon injury and tendinopathy in animal models showed satisfactory outcomes, which enhanced osseointegration, biomechanics, and histology [77–79], which is a promising therapeutic strategy for ACLR.

3.2. Differentiation Induction

3.2.1. Biologic Factors. It is a consensus that growth factors could regulate cell proliferation, ECM elaboration, neovascularization, and mechanical properties. Hence, knowing the exact signaling mechanisms involved in ligament development and repair are essential for improving ACL regeneration, but our current knowledge is much limited and further research needs to be done. Functionally, it has been empirically shown that various growth factors exert positive effects on ligament tissues. Such as transforming growth factor (TGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF), with all having been proven to increase cell proliferation, fibroblastic differentiation, and ECM deposition. As a combination of these factors, PRP could induce mass release of growth factors within one hour following intra-articular administration, which seems a convenient and efficient tool, but related meta-analysis studies found no significant benefit for ACLR in the clinic [80, 81].

Teng et al. found that PRP promoted BMSC osteodifferentiation *in vitro*. Moreover, PRP+BMSCs yielded better tendon-bone healing in rabbits [82]. Single growth factor, such as VEGF [83], also achieved good outcomes. To maintain the effects of these cytokines, gene therapy is a good solution. Runx2 gene upregulated the expression of osteogenic markers and enhanced tendon-bone healing with more new bone tissue formation, without heterotopic ossification [84]. The same results were achieved with BMP2, bFGF, TGF, VEGF, and PDGF gene transfection. Cotransfection of multiple genes is more powerful and efficient for osteogenic differentiation rather than either single gene therapy in Chen et al.'s study [85].

3.2.2. Mechanics. Mechanical loading has been demonstrated to influence cell proliferation, differentiation, apoptosis, and ECM production without growth factors [86–88]. In fibroblasts, mechanical stimulus has been shown to increase cell proliferation, and ECM deposition [89]. It improves tendon-bone healing after ACLR by increasing the amount of fibrocartilage and mechanics. *In vitro*, BMSC/TC coculture stimulated by mechanical stretch showed higher expression levels of collagen I/III, alkaline phosphatase, osteopontin, and tenascin C [90], as well as BMSC alone [88]. In fact, the time, direction, magnitude, and frequency of mechanical stimulation would all influence the cell condition. Early mechanical loading on MSCs inhibited the expression of collagen type I, collagen type II, and fibronectin but enhanced these during the proliferation stage [91]. 8% but not 4% cyclical strain on ligament fibroblasts resulted in better proliferation and collagen production [92]. But it is difficult to control these mechanical parameters *in vivo*, so we need further investigations of the cultured environment before implantation. These could explain how prolonged immobilization would result in the mechanics of damage within the clinic [93].

3.2.3. Biomaterials. In tissue engineering, cell differentiation can be induced by growing the cells on scaffolds with specific composition, architecture, and physicochemical and mechanical properties. Biomaterials not only play a load-bearing role in ACL reconstruction but is also a differentiation inducer.

In native ACL, type I collagen constitutes roughly 90% of the tissue volume, so the use of collagen-based scaffolds has been extensively investigated. Collagen could promote tenogenic differentiation induction, and the collagen-induced tenogenic cells could then arrest osteogenic differentiation mediated by paracrine signals [94]. But immunogenicity and low mechanical strength often limit the application of collagen-based scaffolds. Similar to collagen, silk is a natural biologic material with good tensile strength and biodegradation, but its limited cell adhesion requires some special modification, such as with arginine–glycine–aspartic acid. Silk scaffolds have also been shown to support BMSC attachment and proliferation within a three-dimensional environment and can induce synthesis of fibroblastic markers upon the application of dynamic mechanical loading [95]. Moreover, the hydrophilic properties of silk also influence the proliferation of seeded cells [96]. Electrospinning is a popular and simple technique for fabricating scaffolds with fiber diameters in the nanometer to micron range. Studies showed good capacity of polymer material-based electrospun fibers in promoting tendon fibroblast and MSC proliferation, as well as ECM deposition [97, 98]. Various mechanical parameters of different materials may affect the differentiation of stem cells, such as elastic modulus [99, 100], hydrophilicity or hydrophobicity [101], and substrate topography [102]. Stem cells seeded on aligned nanofibers displayed a more elongated shape with more Scx and ECM marker expression than randomly oriented nanofibers [102, 103]. Graphene-quantum dots could promote MSC osteogenic and adipogenic differentiation [104].

So far, silk scaffold, electrospun scaffold, and decellularized allograft with BMSCs have demonstrated good osseointegration capacity [105–107]. To simulate the insertion stratified structure better, a triphasic silk-based graft was established with BMSCs, chondrocytes, and osteoblasts seeded on different areas of the graft [42]. More novel materials combined with biologics are gaining in popularity.

3.3. Cell Fate. The fate of implanted stem cells remains controversial. Ju et al. used the fluorescent marker Dil dye to track implanted sMSCs, which initially stayed at the tendon-bone interface, and then differentiated into fibroblasts, with the potential of producing collagen fibers or secreting various cytokines for collagen fiber synthesis. But DiI-labeled cells could no longer be observed after 4 weeks [108]. There are three plausible reasons to explain this: missing label, cell replacement, or apoptosis. Lui et al. used the grafts wrapped with the GFP-TDSC sheet for ACLR, but only

few GFP+ cells could be detected at the tunnel interface and the intra-articular graft midsubstance, with the cell number reducing with time [59]. Takeuchi et al. used engineered Tg pigs to track how endogenous cells infiltrate into the graft [12]. The graft was first surrounded by synovia-like tissue with fluorescence at first, then a large number of metabolically active oval cells infiltrated the peripheral region of the graft, resulting in a shift to an equal distribution of oval and spindle-shaped cells. Eventually, spindle-shaped fibroblast-like cells were uniformly distributed, resembling the natural ACL histology.

In some ACL injury models, exogenous cells were detected in the synovium, injured ACL, meniscus, cartilage of femoral condyles, and myotendinous junction of the quadriceps [109, 110]. Transplanted MSCs may produce growth factors such as PDGF, bFGF, and TGF-β, which promote native ACL cell proliferation and migration [111]. Maerz et al. found that tail-injected circulating MSCs preferentially migrate to the synovium of the injured joint, with the upregulation of SDF-1 (chemokines) in the synovial fluid. However, MSC did not enter the intra-articular tissues [110].

The objective of these studies was to form a normal insertion structure, but the source of newly formed fibrocartilage cells remains a mystery. Due to differentiation of the original cells, whether these are derived from transplanted exogenous stem cells or recruited endogenous cells remains ambiguous. How do these MSCs differentiate into fibroblast or other lineages? Tracking of cell fate needs to be more rigorously investigated.

4. Clinical Applications

The search in electronic databases such as PubMed, Embase, and Cochrane Library resulted in 9 clinical trials (Figure 3), including 5 ongoing trials and 4 completed trials [112–115] (trial details see Appendix), highlighting the ongoing evolution of this field.

Based on the published results of completed clinical trials, the overall outcome was quite disappointing (Table 3). Wang et al. showed that the injection of allogeneic BMSCs after ACL reconstruction is safe and tolerable, improving the symptoms and delaying the progress of OA [112]. However, Silva et al. found no significant acceleration in tendon-bone healing with MRI [114]. Alentorn-Geli et al. utilized ADSCs in 20 soccer players with ACL reconstruction and found no statistically significant difference compared to ACLR alone, with respect to pain, biomechanical functions, and MRI scores [115]. Park et al. [116] conducted a 2-year follow-up with patients with hUCB-MSC augmentation and found no statistical differences in biomechanical functions, arthroscopic findings, or tunnel enlargement. Additionally, a noncontrolled trial utilizing autologous bone marrow aspirate combined with PRP and platelet lysate found safe outcomes with MRI and evaluation of clinical function [113].

5. Prospects

5.1. Cell Transplantation. Though preclinical studies have shown promising outcomes, the general clinical effects of stem cells on ACL graft regeneration are controversial, and the heterogeneity of transplanted stem cells needs to be further investigated with more high-quality research studies needed for an accurate and comprehensive conclusion. The clinical application of stem cells is a complex process, in view of the host tissue environment, time, cell adhesion, and dose. Due to the poor blood supply and insufficient nutrition provided by the articular cavity, too much cell injection will lead to necrosis, while too little will not yield a satisfactory effect. Dose- and time-dependent clinical research studies need to be carried out.

The timing of injecting or transplanting stem cells requires further consideration. Based on the process of graft regeneration as described above, two different therapeutic strategies for utilizing stem cells in ACL regeneration have been proposed: (a) during the early stage, transplanted stem cells make up for the hypocellularity, and the immunoregulatory property of MSCs (especially BMSCs) reduces inflammation reaction, as well as facilitate recruitment/activation of endogenous stem cells. Macrophages would accumulate for repair at the tendon-bone interface but often result in the formation of a scar tissue rather than normal insertion site [117], and so the regulation of macrophages by stem cells can enhance tendon-bone healing; (b) the application of stem cells during the remodeling stage may avoid apoptosis of transplanted cells due to poor blood supply. After angiogenesis and ECM deposition, the inner environment may be more suitable for stem cells to implant during the noninflammatory stage [118], but formed ECM may block the implanted cells to migrate towards the inner tendon. Additionally, abundant growth factors such as bFGF, TGF-β1, and PDGF [22] have demonstrated potent effects on tenogenic differentiation induction [119–121].

Different delivery methods have their own pros and cons. To attract stem cells into the scaffold, chemokines can be applied [65]. To simulate the insertion stratified structure, a triphasic silk-based graft was established with different cell types [42, 122], with specific induction treatment being applied to different parts of the graft. Decellularized allogenic scaffold is more similar to the original environment and enables easy seeding of cells [107]. To solve the problem of biocompatibility, biodegradability, and immunogenicity, cell sheet is a new option, which can be harvested from temperature-responsive culture dishes, and it has indeed shown promising outcomes in animal studies [59, 66]. In the clinic, due to the limitations of biomaterial approval, most trials deliver stem cell via injection, which often results in substantial loss of MSCs. Grafts wrapped in stem cell collagen seem a safe and simple solution. In summary, a carrier with great natural biodegradability, cell adhesion, biomechanics, biocompatibility, and insertion spatial simulation is required, and silk-based scaffolds have shown promising potential.

5.2. Differentiation of Stem Cells into Tendon/Ligament Cell Lineages. In embryos, tendon development requires both physiological and biomechanical stimulation [123, 124]. Temporal coordination of various physiological signals at early developmental stages, such as TGF-β, BMP, and FGF

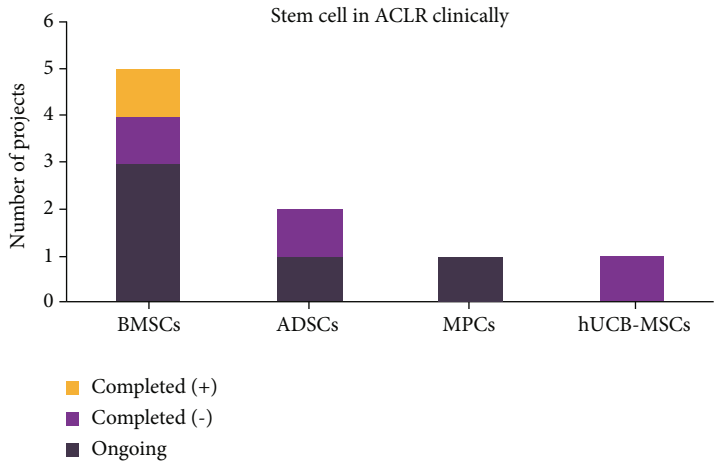

Figure 3: The number of ongoing and completed clinical projects with positive or negative results in the application of different stem cell lineages after ACL reconstruction. BMSC: bone marrow-derived mesenchymal stem cells; MPC: mesenchymal precursor cell; ADSC: adipose tissue-derived stem cell; hCDB-MSC: human cord blood-derived mesenchymal stem cell.

Table 3: Published clinical trials of stem cell therapy for ACL graft regeneration.

Author	Cell resource	Patient*	Follow-up	Evaluation	Outcome	Other outcomes
Wang et al. [112]	BMSCs	11 vs. 6	2 y	Adverse event; pain; function; MRI; LifeQ	+	Less pain, symptoms, bone expansion, joint space narrowing, and cartilage volume loss
Silva et al. [149]	BMSCs	20 vs. 23	1 y	MRI	-	No signal-to-noise ratio difference
Alentorn-Geli et al. [115]	ADSCs	20 vs. 19	1 y	Pain; function; MRI	-	
Park et al. [116]	hUCB-MSCs	10 vs. 10 vs. 10#	2 y	Adverse event; KT; function; arthroscopy	—	Safe but no clinical advantage

*The experimental group (ACLR+stem cell) vs. the negative control group (ACLR); #the experimental group (ACLR+stem cell+HA) vs. the negative control group (ACLR) vs. the positive control group (ACLR+HA).

[125–127], as well as biomechanical stimulation at later stage [123], promotes tenogenic differentiation. The origin of ACL is still under research. Most joint tissues derive from GDF5(+) mesenchymal cell [128], of which Lgr5 +/Scx+/Col22a1- interzone cells are restricted within the ligament lineage [129]. Scx+/Sox9+ precursors are also considered as the origin of the ACL [130], although exact signaling mechanisms involved in ligament development are still unclear. Several markers of embryonic tendon development were identified, but these do not provide functional properties. Based on embryonic tendon development, step induction is a logical method for simulating the development of tenocytes, with enhanced self-renewal, and long-term viability. Chen et al. induced hESCs to differentiate into MSCs and subsequently allow the MSCs to form tendon-like tissues with mechanical stress *in vitro* and *in vivo* [131].

Learning from embryonic tendon development can improve tendon tissue engineering strategies with adult stem cells, and tenogenic cues and markers will need to be established for step-wise induction [132]. Some studies have delivered MSCs together with exogenous proteinogenic growth factors to induce tenogenic differentiation. TGF is considered as an inducer of the tendon transcription factor Scx [70, 127], which can direct MSC differentiation towards the tenogenic lineage [119]. FGF mainly promotes matrix production for tendon maturation [120], and FGF4 treatment has been shown to significantly downregulate the gene expression levels of all tendon markers (Scx, TGFβ2, Tnmd, Col I, and elastin) in MSCs but can only downregulate the mRNA levels of elastin in TDSC [121]. The BMP family of growth factors is essential to both osteogenic and chondrogenic differentiation [126], which may activate cytoskeletal reorganization or the Smad signaling pathway [133, 134]. In particular, BMP-12/13/14 signaling has been shown to be proteinogenic [135]. CTGF also plays an auxiliary role during tenogenic differentiation, by activating Scx, Tnmd, and other ECM marker expression, inducing fibroblastic effect and ECM production [136, 137]. In addition, Wnt signal was found to induce Tnmd expression in BMSCs via glycogen synthase kinase-3 [138]. These signaling factors play key roles in tendon differentiation and regeneration.

All stem cell therapies have the inherent risk of tumorigenicity, due to the aberration of chromosomal, copy number, and single nucleotide, hindering clinical translation [139, 140]. Hence, some researchers have turned to exosomes as an alternative, the specific vesicles secreted by stem cells, which can directly deliver the bioactive factors with low risk

of tumorigenicity and undesired spontaneous differentiation. A similar tool is conditioned medium (CM), which represents a mixture of different factors secreted by the cells. The application of BMSC-CM accelerates graft-bone incorporation and midsubstance ligamentization and enhances differentiation as well [141]. These cell-free preparations have the advantages of less ossification, less calcification, and easy restoration, with various different proteins, nucleic acids, and lipid components being linked to their potency.

5.3. Current Challenges in Stem Cell Therapy for ACLR. Although challenges exist, preclinical evidence predicts a promising future for stem cell approach to ACLR, despite most (3/4) clinical research studies showing controversial outcomes. Currently, there are several ongoing human clinical trials in this area. Due to few studies on stem cell therapy for ACLR, we are unable to conduct a deep meta-analysis in this systematic review. In general, how exactly stem cells participate in human ACL regeneration and whether it has clinical benefits will require further study.

In addition, because stem cell transplantation is a biological therapeutic strategy, the stability and oncogenicity of stem cells require consistent long-term safety verification. The published scientific literature confirms the short-term (<24 months) safety and tolerance of stem cells in ACLR, but the implanted cells need long-term tracking, which has been poorly studied to date.

Third, the choice of the stem cell source is another important consideration. Stem cells derived from different sources all showed good capacity in promoting regeneration, but their relative effects need to be compared to optimize the therapeutic efficacy. With respect to availability and ease of isolation, ADSCs and BMSCs may have advantages over other stem cell types. In terms of proliferative capacity and ligamentous differentiation potential, TDSC/LDSC is regarded as having the most potential, but limited cell quantity may limit clinical applications. Proper differentiation of alternative stem cell lineages either *in vitro* or *in vivo* will be particularly crucial, because they are capable of differentiating into multiple tissue types. Current applications in humans are at the primary stage, so the differentiation induction *in vivo* is not mature and safe. Moreover, the implantation methodology and cell fate have been discussed previously, including the dose, time, supplementary agent, and material.

6. Conclusion

Almost all utilized stem cell lineages showed good capacity in promoting tendon-bone regeneration in animal models. Among the various different stem cell types, BMSCs are most commonly investigated, while LDSC/TDCS showed better potential for tendon/ligament lineage-specific differentiation. With differentiation inducers, such as growth factors, mechanical stimuli, and biomaterials, stem cells have better capacity to differentiate into ligament, fibrocartilage, and bone, as well as regulate inflammation through paracrine pathways, promoting graft regeneration. The application of stem cells in the clinic often results in disappointing outcomes and needs further investigations.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

The detail of clinical trials in the application of different stem cell lineages after ACL reconstruction. Description: a comprehensive search of the literature was carried out in Sep. 2nd, 2020, using electronic databases PubMed, Embase, and Cochrane Library. The keywords "anterior cruciate ligament" and "stem cell" were combined without language or time restrictions. The results were limited to RCTs. *(Supplementary Materials)*

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Review Article

The Crosstalk between Mesenchymal Stem Cells and Macrophages in Bone Regeneration: A Systematic Review

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Bone regeneration is a complex and well-coordinated process that involves crosstalk between immune cells and resident cells in the injury site. Transplantation of mesenchymal stem cells (MSCs) is a promising strategy to enhance bone regeneration. Growing evidence suggests that macrophages have a significant impact on osteogenesis during bone regeneration. However, the precise mechanisms by which macrophage subtypes influence bone regeneration and how MSCs communicate with macrophages have not yet been fully elucidated. In this systematic literature review, we gathered evidence regarding the crosstalk between MSCs and macrophages during bone regeneration. According to the PRISMA protocol, we extracted literature from PubMed and Embase databases by using "mesenchymal stem cells" and "macrophages" and "bone regeneration" as keywords. Thirty-three studies were selected for this review. MSCs isolated from both bone marrow and adipose tissue and both primary macrophages and macrophage cell lines were used in the selected studies. In conclusion, anti-inflammatory macrophages (M2) have significantly more potential to strengthen bone regeneration compared with naïve (M0) and classically activated macrophages (M1). Transplantation of MSCs induced M1-to-M2 transition and transformed the skeletal microenvironment to facilitate bone regeneration in bone fracture and bone defect models. This review highlights the complexity between MSCs and macrophages, providing more insight into the polarized macrophage behavior in this evolving field of osteoimmunology. The results may serve as a useful reference for definite success in MSC-based therapy based on the critical interaction with macrophages.

1. Introduction

1.1. Fracture Healing and Bone Regeneration. Currently, over 20 million people suffer from fractures annually, predominantly due to the prevalence of osteoporosis, osteosarcoma, osteomalacia, osteomyelitis, and atrophic nonunion. Only one-quarter of these patients have received orthopedic interventions, of which more than half were treatments like bone grafting, which target the afflicted sites [1, 2]. However, the high recurrence imposes a severe economic burden on the healthcare system. To address this health problem, numerous researchers have investigated the bone regeneration process and intervention in hopes of finding more effective ways to treat these injuries.

Fracture healing is a complex and well-orchestrated process to develop the bone matrix in defective sites without forming fibrous scars, involving a series of extracellular and intracellular signaling pathways. Fracture healing can be

characterized as two types: primary bone repair (direct) and secondary bone repair (indirect) [3]. Primary fracture repair does not typically occur naturally as it only occurs with rigid fixation of bone ends, direct contact, and absolute stability. On the other hand, secondary fracture repair, consisting of endochondral and intramembranous ossification, is the most common process of fracture healing and can be enhanced by load bearing and micromotion. Acute inflammatory responses within the fracture site are necessary to initiate tissue regeneration, accompanied by the secretion of proinflammatory molecules during secondary fracture repair. Biological events such as the recruitment of inflammatory cells and the promotion of angiogenesis occur after the secretion of those proinflammatory molecules. Endogenous MSCs, recruited from local soft tissues and bone marrow, migrate toward the injury site, proliferate, and differentiate into osteogenic cells. Cartilaginous callus formation provides the stable structure of the fracture site which will be replaced by a hard bony callus with more mechanical rigidity via mineralization and resorption of the soft callus. Revascularization and neoangiogenesis are also essential for fully restoring the biomechanical properties of bone [4].

1.2. Osteoimmunology in Bone Healing: The Role of Macrophages in Bone Healing. The entire process of fracture healing can be roughly divided into two stages: the early inflammatory phase and the tissue regeneration phase. In secondary bone repair, immune cells infiltrate the hematoma and release cytokines to initiate inflammation that is accompanied by short-lived but extensive effects on endogenous MSC recruitment and subsequent regenerative processing. Although various types of immune cells are involved [5, 6], macrophages exhibit inseparable cooperation with osteolineage cells during the whole spectrum of the fracture healing process.

Macrophage ablation reduces bone mineral density and decreased trabecular numbers during the early stage of skeletal development [7]. Schlundt et al. [8] also revealed the role of macrophages in both endochondral ossification and intramembranous ossification. Disturbed endochondral ossification due to defective cartilage resorption was observed in mice with selective macrophage depletion; meanwhile, enhanced periosteal bone formation was observed in the region distant from the fracture gap. The necessity of macrophages in both initiation and progression of early endochondral ossification was evident in a macrophage Fas-induced apoptosis (MAFIA) model [9].

Although macrophages are identified as one of the first infiltrating cells during fractures with a proinflammatory status, they also significantly regulate subsequent bone repair. Different subtypes of macrophages correspond to the stage of fracture healing. In the inflammatory phase, classically activated M1 macrophages, hereafter M1, perform phagocytosis and produce proinflammatory cytokines, such as TNF, IL-1 beta, IL-6, and IL-12, to promote osteogenesis in early and middle stages without enhancing matrix mineralization [10, 11]. In the late stage, alternatively activated macrophages, hereafter M2, release proregenerative cytokines, such as IL-10, TGF-beta, BMP2, and VEGF, to build up an anti-inflammatory environment and facilitate osteochondral differentiation and angiogenesis [5, 10]. Since both subtypes of macrophages make substantive contributions in different stages of fracture healing, regulating the presence of different macrophage subtypes is considered a therapeutic approach for fracture healing.

1.3. Crosstalk of Mesenchymal Stem Cells and Macrophages in Bone Healing. MSCs are regarded as a promising bioagent for treating various diseases based on their immunoregulatory capacity [12, 13]. Interestingly, the presence of macrophages is involved in the therapeutic effects of MSCs. The communication between MSCs and macrophages has been extensively studied [14]; the secretome of MSCs is altered in response to inflammatory macrophages, while a corresponding reaction of macrophages following MSC therapy is also observed—forming a feedback loop. With the emphasis on fracture healing and bone regeneration, the interaction of macrophages and MSCs has been recently summarized by Pajarinen et al., showing paracrine molecules derived from macrophages play critical roles in guiding MSC differentiation [11]. A number of reviews and systematic reviews have emphasized the role of MSCs [15–18] and macrophage polarization [19–21] in bone regeneration. However, the comprehensive understanding of the communication between MSCs and macrophages during bone regeneration remains insufficient. This review is aimed at thoroughly and systematically analyzing the communication between MSCs and macrophages in order to fill the knowledge gap of this unclarified phenomenon during bone regeneration.

2. Methods

2.1. Search Strategy. A systematic review was conducted to systematically assess articles on the crosstalk between MSCs and macrophages in bone regeneration. PubMed and Embase databases were comprehensively used to search for relevant literature by two investigators (LY Shin, HT Xu). The search term keywords are "mesenchymal stem cells" AND "macrophages" AND "bone regeneration," combing with the mesh terms of these keywords. The details of the entire search terms and the searching workflow by PRISMA can be referred to Appendixes A–C.

2.2. Inclusion and Exclusion Criteria. Eligibility screening of titles and abstracts was conducted based on the following criteria: (1) articles are in English and were published in the last 10 years; (2) primary studies must be related to "mesenchymal stem cells" and "macrophages" and "bone regeneration"; and (3) review articles, case reports, letters, editorials, and correspondences were all excluded.

2.3. Data Extraction and Management. A standard process for data extraction of each eligible article was performed. Titles not relevant to the topic were removed first, followed by the exclusion of studies with irrelevant abstracts. All duplicates were removed. The following information was summarized from the selected studies: (1) authors, (2) cell

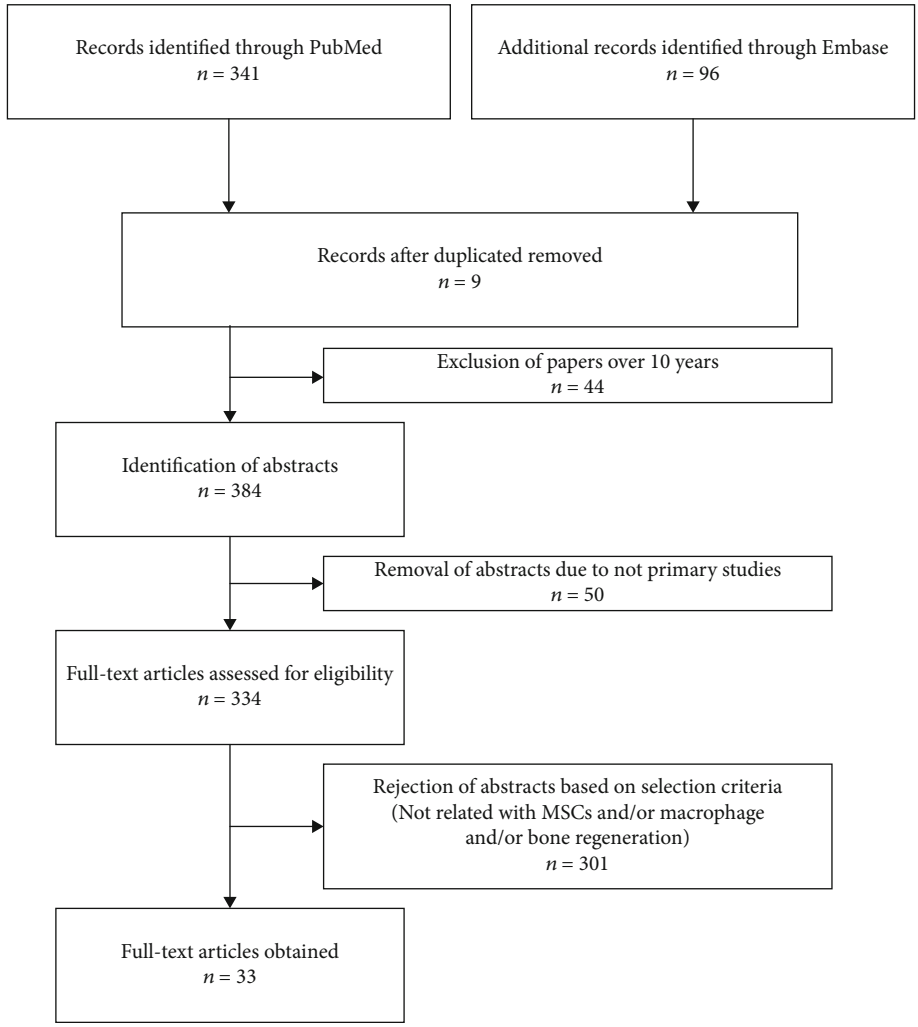

Figure 1: Flow diagram of the systematic review on the crosstalk of MSCs and macrophages. A total of 437 studies were retrieved based on the search strategy mentioned in the methods. Nine records after duplicates were removed. 44 works of literature published more than 10 years were excluded. After reviewing the titles and abstracts, 50 records were removed because the studies were not primary studies. After reviewing the titles and abstracts, 301 records were removed because the studies did not match the selection criteria. Finally, 33 studies met the inclusion criteria and were selected for this systematic review.

source, (3) study type, (4) cell management, (5) interaction between MSCs and macrophages, and (6) proposed mechanisms. If there was any uncertainty or inconsistency between the reviewers (LY Shin, HT Xu), a third reviewer was consulted (CW Lee) with final identification.

2.4. Quality Assessment. The quality of selected papers was evaluated with a quality system constructed by Wells and Littell [22] (Appendix D). The following 8 questions were adopted in the quality scoring system. Was the study hypothesis/aim/objective clearly described? Were the experimental designs for the study well described? Were the method and materials well described? Were the time points of data collection clearly defined? Were the main outcome measurements clearly defined? Were the experimental groups well compared with the control group? Were the results well described? Was the limitation of the article discussed? Regarding each question, 1 point was allocated for "yes" and 0 points were allocated for "no." A sum of the scores for each study was calculated independently, with a total score out of 8. Quality assessment was graded by the scores. Six to 8 was considered excellent, 4 to 6 was considered good, 2 to 4 was considered poor, and 0 to 2 was considered bad. Detailed score evaluation of selected studies can be referred to Appendix E.

3. Results

3.1. Search Results and Characteristics. 437 articles were identified in the primary searches. Two reviewers independently assessed the articles according to the inclusion and exclusion criteria to minimize bias and advance the strength of the selected articles. A joint discussion was conducted by a third reviewer when differences emerged during the assessment. After full articles were retrieved, a total of 33 studies were selected for data extraction in this review. Details of the selecting process are shown in Figure 1.

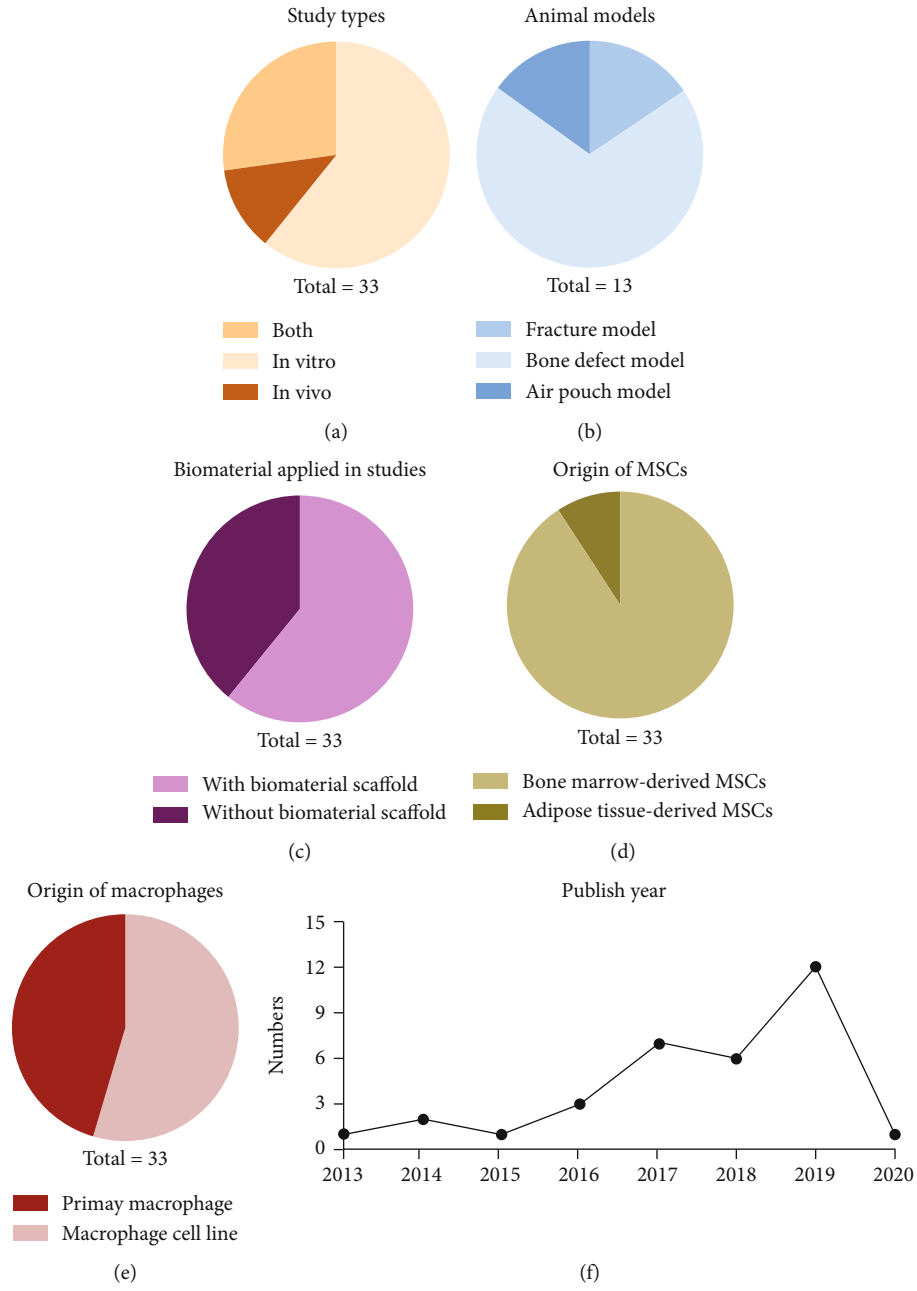

Figure 2: Study characteristics of the systematic review. (a) Categories of experiments. (b) Animal models of the in vivo studies. (c) The origin of the MSCs applied in studies. (d) The origin of macrophages applied in studies. (e) The proportion of biomaterials used in studies. (f) Published year of selected studies. Database searching and study identification in this review are till Jan of 2020.

All studies were published between 2013 and 2020. The categories of experiments present that 20 articles were in vitro studies, 4 articles were in vivo studies, and 9 articles applied both the in vitro and in vivo assessments. 20 articles applied biomaterial scaffolds and MSCs for bone regeneration. Among the 13 animal studies, 9 studies were using the bone defect model, 2 studies were using the fracture model, and 2 studies were using the air pouch model. MSCs derived from bone marrow were applied in whole articles, except one article that used the adipose-derived MSCs. Macrophages used in experiments can be divided into two major categories: (1) primary macrophages derived from humans or animals (mouse, rat, and rabbit) and (2) macrophage cell lines (RAW 264.7 and THP-1). Study characteristics mentioned above are summarized in Figure 2. We classified these articles into two subgroups: (1) the immunoregulatory potential of MSCs on macrophages in bone regeneration and (2) the effects of macrophages on MSC osteogenesis. Supplemental details of the experiments can be referred to Appendix F.

3.2. Immunoregulatory Potential of MSCs on Macrophages in Bone Regeneration. The immunomodulatory capability of MSCs and relevant effects on macrophage polarization are further discussed within this section, accompanied by

the follow-up performance in bone regeneration in both the in vivo and in vitro models. Detailed results are listed in Table 1.

To uncover the subtypes of macrophages affected by exogenous MSCs, Seebach et al., Tasso et al., and Tour et al. implanted MSCs using fibrin carriers or hydroxyapatite scaffolds into bone defects. M1 macrophages and endothelial progenitor cells served as primary invaders of the bone defect site after MSC implantation in the first 2 weeks, while only a few M2 macrophages existed in the cell infiltrated area [23, 24]. M1-to-M2 macrophage switching induced by implanted MSCs has been observed in late-stage bone healing, which demonstrates that M2 macrophages prefer to accumulate in the front of cell-dense migration sites and have a proresolving phenotype that recruits vasculogenic and osteogenic progenitors from bone marrow. This M2 polarization was attributed to exogenous MSC-secreted PGE2 activating the NF-κB pathway [25]. M1-to-M2 transitions are not only sequential but also closely associated with the healing process. M1-to-M2 transition was also found in Li et al.'s study which applied an osteogenesis-inducing material, laponite (Lap), in bone defects. Although Lap is beneficial for bone regeneration, as a foreign object, it is still associated with inflammation. They found that MSCs converted laponite-(Lap-) induced M1 macrophages into the M2 phenotype, creating an anti-inflammatory/prosolving environment that promotes osteogenesis [26]. Nevertheless, the transplanted MSCs cannot be detected at 4 weeks posttransplantation, suggesting MSCs might regulate macrophage polarization during the early stage [23, 24].

MSC-induced M2 polarization is described in vitro as well. MSCs and macrophages cocultured with 1,25-dihydroxyvitamin D3 supplementation could reduce the secretion of inflammatory factors as a result of MSC-secreted PGE2 and VEGF. The CM from the cocultures further enhanced matrix maturation and mineralization of BMSCs under osteogenic conditions [27]. Preconditioning BMSCs with the combination of LPS and TNF-α was another strategy to affect macrophage polarization. Lin et al. found that PGE2 secreted from preconditioned BMSCs modulates M1 macrophages into an anti-inflammatory phenotype via the NF-κB/COX2 pathway with no influence on mineralization [28]. In He et al.'s study, CM from MSCs cultured on LL-37-loaded silk fibroin nanoparticles (SFNPs) promotes M2 macrophage polarization. The increased IL-4 and TGF-β1 from MSCs cultured on LL-37-loaded SFNPs were regarded as the main cause of M2 polarization [29]. Anti-inflammatory cytokine IL-4 is beneficial for bone formation by enhancing scaffold vascularization and inhibiting osteoclast activation [30–33]. Excess IL-4 produced by genetically modified MSCs is another strategy to improve bone healing. IL-4-secreting MSCs are NF-κB-responsive and continuously produce large amounts of IL-4 to further enhance M1-to-M2 transition. However, the IL-4-secreting MSCs reduced the osteogenic capacity in vitro, suggesting excessive IL-4 leaking into systemic circulation may potentially impair bone formation [34].

Both naïve MSCs and osteogenically differentiating MSCs are capable of altering the phenotypes of macrophages. After treatment with pre-osteoblast-derived exosomes, LPS-induced macrophages showed decreasing proinflammatory gene expression and lower levels of M1 markers. The authors realized that the differentiating MSC secretome could recruit more naïve MSCs to the injury site and produce a positive feedback loop to magnify naïve MSC exosome signals, thereby reducing subsequent inflammation and promoting bone regeneration [35].

In summary, MSC transplantation not only mitigates chronic inflammation but also promotes bone regeneration via M2 phenotype switching. Cotransplantation of MSCs could effectively ameliorate biomaterial-induced foreign body reactions in the bone that is associated with bone regeneration. Most noteworthy is the immunomodulatory effect of MSCs on macrophages. This provides a new insight that bone regeneration can be improved by osteoimmune environment modulation instead of enhancing bone formation through the direct regulation of osteolineage cells.

3.3. The Effects of Macrophages on MSC Osteogenesis. The skeletal and immune systems closely interact with each other by way of common cell precursors and molecular mediators. The different subtypes of macrophages and their influence on MSCs undergoing osteogenic differentiation are discussed in this section. In-depth details and results are listed in Tables 2 and 3.

3.3.1. Bone Regeneration Enhanced by M1 Macrophages. Enhanced osteogenic differentiation of MSCs and bone regeneration have been observed in the proinflammatory environment, which is built by M1 macrophages. The macrophage cell line RAW 264.7 cultured with mesoporous silica nanospheres (MSNs) or graphene oxide (GO) increased the amount of proinflammatory cytokines (TNF-α, IL-6, IL-1β, and IFN-γ) and OSM. This inflammatory environment stimulated osteogenic differentiation of MSCs through OSM and NF-κB pathways [36, 37]. Furthermore, Cu-MSN/macrophage CM upregulated OPG and downregulated RANKL in BMSCs to suppress osteoclastogenesis [36]. In coculture experiments, carbon nanohorn- (CNH-) engulfed macrophages also expressed OSM to accelerate osteogenic differentiation of MSCs via the STAT3 signaling pathway [38]. Lu et al. demonstrated that LPS-induced M1 macrophages promote osteogenesis via the COX2-PGE2 pathway. Increasing the ratio of M1 macrophages/MSCs in coculture to mimic the inflammatory reaction at the fracture site could further promote osteogenesis. However, OPG produced by MSCs was negatively regulated by LPS-induced M1 macrophages after coculture, suggesting the significance of the OPG-RANKL ratio and its relation to the role of M1 macrophages in modulating osteoclastogenesis need further investigation [39]. Tu et al. provided another perspective to explain the stimulatory effects of proinflammatory macrophages on MSC osteogenesis. IL-23 secretion from macrophages directly induced osteogenesis of MSCs by activating

Table 1: Immunoregulatory potential of MSCs on macrophages in bone regeneration.

Author	Cell source	Study type	Cell management	Immunoregulatory potential of MSCs on Mφs	Proposed mechanisms
Tasso R 2013	C57BL/6 mice—BMSCs; C57BL/6 mice—Mφs	In vitro & in vivo	In vitro: Mφs cultured in the IL-1a-stimulated BMSC-CM In vivo: BMSCs seeded on bioceramic scaffolds are transplanted	In vitro: the percentage of M2 Mφs significantly increases after Mφs cultured in the CM from BMSCs In vivo: implanted BMSCs induce Mφ switching to a proresolving phenotype and recruit vasculogenic and osteogenic progenitors from BM	PGE2 secreted from BMSCs activates the NF-κB pathway to affect M2 Mφ polarization
Seebach E 2014	SD rats—BMSCs; SD rats—Mφs	In vivo	BMSCs embedded in a fibrin carrier are implanted into femoral bone defects	BMSC composites attract proinflammatory M1 Mφs and endothelial progenitors and then promote implant integration, angiogenesis, and tissue maturation	/
Tour G 2014	Lewis GFP transgenic rat—BMSCs; SD rats—Mφs	In vivo	BMSCs with HA-ECM are implanted into calvarial bone defects	M1 Mφs were prevalent than M2 Mφs in the calvarial defects at 2 weeks after surgery	/
Lin T 2017	C57BL/6 mice—BMSCs; C57BL/6 mice—Mφs	In vitro	Mφs are treated with the CM from LPS-exposed $MSC^{NF\text{-}\kappa BREIL4}$	CM from $MSC^{NF\text{-}\kappa BREIL4}$ modulates inflammatory M1 Mφs into an anti-inflammatory M2 Mφs	NF-κB-sensing $MSC^{NF\text{-}\kappa BREIL4}$ produces excessive IL-4 for immunomodulation
Lin T 2017	C57BL/6 mice—BMSCs; C57BL/6 mice—Mφs	In vitro	Preconditioned BMSCs with LPS plus TNF-α culture with M1 Mφs	Preconditioned BMSCs modulate M1 Mφs into an anti-inflammatory phenotype and increase PGE2 production but not affect mineralization	Preconditioned BMSC-secreted PGE2 can be stimulated by TNF-α through the NF-κB/COX2-dependent pathway
Saldana L 2017	Human—BMSCs; THP-1—Mφs	In vitro	BMSCs undergo osteogenic differentiation with the CM from the cocultures of BMSCs, Mφs, and 1,25D3	1,25D3 promotes the switching of cocultured Mφs toward the M2 phenotype secreting anti-inflammatory factors (IL-10, PGE2) to enhance matrix maturation and mineralization of BMSCs	/
Li T 2018	SD rats—BMSCs; RAW 264.7—Mφs	In vitro & in vivo	In vitro: Lap/Mφ CM with osteogenic components is applied to stimulate BMSCs In vivo: Lap+BMSCs are injected into the bone defect	In vitro: BMSCs reversed M1 Mφs induced by Lap into M2 Mφs and promoted osteogenesis In vivo: the Lap+BMSC group shows obvious new bone formation with a significant increase in M2 Mφs	Activation of the OSM pathway is likely involved in the enhanced osteogenesis by BMSCs
He Y 2019	SD rats—BMSCs; RAW 264.7—Mφs	In vitro & in vivo	In vitro: CM from BMSCs seeded on Ti-SF/LL-37 is applied on Mφ culturing In vivo: LL-37-loaded SFNPs of Ti rods are inserted into the bone defect	In vitro: M2 phenotype switching of Mφs is induced by the BMSCs seeded on Ti-SF/LL-37 In vivo: demonstrated in Table 2	/
Wei F 2019	Human—BMSCs; RAW 264.7—Mφs	In vitro	LPS-induced Mφs are treated with exosomes first isolating from osteogenically differentiating BMSCs	The uptake of exosomes significantly decreases the M1 phenotypic marker of LPS-induced Mφs	/

BMSCs: bone marrow stem cells; Mφs: macrophages; CM: conditioned medium; PGE2: prostaglandin E2; NF-κB: nuclear factor-kappa B; GFP: green fluorescent protein; HA-ECM: hydroxyapatite-extracellular matrix; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor-alpha; COX2: cyclooxygenase 2; 1,25D3: 1,25-dihydroxyvitamin D3; Lap: laponite; OSM: oncostatin M; Ti-SF: titanium-silk fibroin; SFNPs: silk fibroin nanoparticles.

Table 2: Involvement of M1 macrophages in MSC osteogenic differentiation and bone regeneration.

Author	Cell source	Study type	Cell management	Involvement of Mφ polarization in MSC osteogenic induction	Proposed mechanisms
Tu B 2015	Human—BMSCs; THP-1—Mφs	In vitro	Mφ CM collected for treating BMSCs under osteogenic induction conditions	IL-23 secretion from proinflammatory Mφs promotes the osteogenesis of BMSCs	Mφ-secreted IL-23 activates the STAT3 and β-catenin signaling and thus promotes the osteogenic differentiation of BMSCs
Hirata E 2016	Human—BMSCs; human—Mφs	In vitro	Coculture of BMSCs and Mφs in the presence of CNHs	ALP activity is increased under the coculture of Mφs and MSCs in the presence of CNHs	OSM from activated Mφs induces osteoblast differentiation and matrix mineralization through STAT3
Shi M 2016	Human—BMSCs; RAW 264.7—Mφs	In vitro	BMSCs cultured in Cu-MSN/Mφ CM under osteogenic differentiation	Mφs phagocytize Cu-MSNs and produce proinflammatory cytokines leading to better osteogenic differentiation of BMSCs	Cu-MSN/Mφ CM enhances the osteogenic differentiation of BMSCs through the activation of the OSM pathway
Lu LY 2017	C57BL/6 mice—BMSCs; C57BL/6 mice—Mφs	In vitro	Coculture of BMSCs and polarized Mφs (M1 induced by LPS and M2 induced by IL-4)	Polarized Mφs enhance bone mineralization, especially proinflammatory M1 Mφs	M1 Mφs enhance BMSC osteogenesis and bone formation via the COX2-PGE2 pathway
Tang H 2017	Human—ADSCs; THP-1—Mφs	In vitro	3D spheroid cocultures of M1 Mφs and ADSCs are conducted under osteogenic differentiation conditions	The osteogenic differentiation of ADSCs is inhibited by M1 Mφs	N-cadherin-mediated cell-cell interactions between M1 Mφs and ADMSCs result in inhibited osteogenesis
Xue D 2018	Human—BMSCs; RAW 264.7—Mφs	In vitro	BMSCs undergo osteogenic differentiation added with the CM from GO/Mφs	Coculture of GO and Mφs induced M1 Mφ transition and produced proinflammatory cytokines in the CM, further enhancing BMSC osteogenesis	The proinflammatory environment induced by GO promote osteogenic differentiation of BMSCs through OSM and NF-κB pathways
Wasnik S 2018	C57BL/6 mice—BMSCs; C57BL/6 mice—Mφs	In vivo	Mice with the fracture at the midshaft receive a daily s.c. dose of 1,25(OH)2D	The suppression of fracture healing induced by 1,25(OH)2D is mediated by the inhibition of M1 Mφs during the proinflammatory stage	/
Nathan K 2019	C57BL/6 mice—BMSCs; C57BL/6 mice—Mφs	In vitro	Coculture of BMSCs and M1 Mφs in the presence of IL-4 under the osteogenic induction medium	Temporal modulation of M1-to-M2 polarization maximizes MSC matrix mineralization	/
Tang H 2019	Human—ADSCs; THP-1—Mφs	In vitro	M1 Mφs and ADSC coculture on PLGA/PCL scaffolds with osteogenic induction components	M1 Mφs inhibit the osteogenic differentiation of ADMSCs on 3D PLGA/PCL scaffolds	M1 Mφs inhibit osteogenic-related pathways (BMP & OSM signaling) during ADSC differentiation
Y He 2019	SD rats—BMSCs; RAW 264.7—Mφs	In vitro & in vivo	In vitro: CM from Mφs seeded on Ti-SF/LL-37 is applied on BMSC culturing In vivo: LL-37-loaded SFNPs of Ti rods are inserted into the bone defect	In vitro: osteogenic differentiation of BMSCs was enhanced by additional CM from Mφs incubated on Ti-SF/LL-37 In vivo: The Ti-SF/LL-37 group effectively induced both proinflammatory factors and exhibited improved osteogenesis ability	/

BMSCs: bone marrow stem cells; Mφs: macrophages; CNHs: carbon nanohorns; ALP: alkaline phosphatase; OSM: oncostatin M; STAT3: signal transducer and activator of transcription 3; Cu-MSNs: Cu-containing mesoporous silica nanospheres; CM: conditioned medium; COX2: cyclooxygenase 2; PGE2: prostaglandin E2; GO: graphene oxide; NF-κB: nuclear factor-kappa B.

Table 3: Involvement of M2 macrophages in MSC osteogenic differentiation and bone regeneration.

Author	Cell source	Study type	Cell management	Involvement of Mφ polarization in MSC osteogenic induction	Proposed mechanisms
Gong L 2016	C57BL/6 mice—BMSCs; C57BL/6 mice—Mφs	In vitro	Coculture of BMSCs and polarized Mφs (M1 induced by LPS and M2 induced by IL-4) with the osteogenic medium	M2 Mφs enhance osteoblast differentiation of MSCs	Proregenerative cytokines (TGF-β, VEGF, and IGF-1) produced by M2 Mφs facilitate MSC osteogenesis
Chen Z 2017	SD rats—BMSCs; RAW 264.7—Mφs	In vitro	CM from nanopore structure/Mφs is applied to stimulate BMSCs under the osteogenic induction medium	Osteogenesis of BMSCs is enhanced by the stimulation of the nanostructure/Mφ CM	Osteogenic pathways (Wnt and BMP) of BMSCs are regulated by different nanopore-induced inflammatory environments
Zhang Y 2017	Human—ADSCs; THP-1—Mφs	In vitro	Direct and indirect coculture of ADSCs and polarized Mφs during osteogenic differentiation (M1 induced by IFN-γ & LPS and M2 induced by IL-4 & IL-13)	M2 Mφs have beneficial effects on ADSC mineralization by promoting their proliferation and osteogenic differentiation	M2 Mφs enhance osteogenic differentiation of MSCs in a manner dependent on OSM and BMP2 signaling pathways
Tang H 2017	Human—ADSCs; THP-1—Mφs	In vitro	3D spheroid cocultures of M2 Mφs and ADSCs are conducted under osteogenic differentiation conditions	The osteogenic differentiation of ADSCs was inhibited by M2 Mφs	N-cadherin-mediated cell-cell interactions between M2 Mφs and ADMSCs result in inhibited osteogenesis
He XT 2018	C57BL/6 mice—BMSCs; RAW 264.7—Mφs	In vitro	BMSCs incubated with different CMs generated by unpolarized Mφs (M0) or polarized Mφs (M1 and M2) supplemented with osteoinductive media	CM from M2 Mφs exhibits the potential to foster osteogenic differentiation of BMSCs	/
Wang J 2018	C57BL/6 mice—BMSCs; C57BL/6 mice—Mφs	In vitro	BMSCs undergo osteogenic differentiation with NT/Mφ CM	NT-30 induces more M2 Mφs while enhancing BMSC osteogenesis while NT-100 induces M1 Mφ polarization	/
Ma QL 2018	Human—BMSCs; human—Mφs	In vitro & in vivo	In vitro: osteogenic differentiation of BMSCs on different Ti surfaces in CM from Mφs In vivo: three types of Ti implants inserted in the distal femur	In vitro: the NT surfaces and corresponding CM types together promote osteogenic gene expression in BMSCs, and osteoclast formation is likely promoted by factors (sRANKL, OPG, and M-CSF) secreted by BMSCs cultured in NT20-CM but suppressed in NT5-CM In vivo: the NT5 and NT20 surfaces lead to enhanced bone formation after 12 weeks postimplantation	NF-κB and BMP pathways activated by the polarized macrophages are involved in both osteogenesis and osteoclastogenesis

Table 3: Continued.

Author	Cell source	Study type	Cell management	Involvement of Mφ polarization in MSC osteogenic induction	Proposed mechanisms
Jin SS 2019	Human—BMSCs; THP-1—Mφs	In vitro & in vivo	In vitro: BMSCs are cultured with supernatants of Mφs seeded on scaffolds In vivo: deplete the Mφs by clodronate liposomes and implant HIMC as a bone graft in rat mandible defect models	In vitro: M2 Mφ polarization induced by HIMC interacts with BMSCs to promote osteogenic differentiation and mineralization In vivo: the ectopic bone formation stimulated by tricalcium phosphate is blocked by Mφ depletion	HIMC intrinsically promotes M2 Mφ polarization with IL-4 secretion, further enhancing BMSC osteogenesis
Sadowska JM 2019	Human—BMSCs, human—SaOS-2; RAW 264.7—Mφs	In vitro	LPS-stimulated Mφs first cultured on the CaPs and CaP-Mφ-conditioned extracts are incubated with the bone-forming cells (BMSCs and SaOS-2) for osteogenic stimulation	The microenvironment created after culturing Mφs on CDHA showed more potent osteogenic effects, fostering osteogenic differentiation of both BMSCs and SaOS-2 cells	/
Tang H 2019	Human—ADSCs; THP-1—Mφs	In vitro	Mφs (M1, M2) and ADSC coculture on PLGA/PCL scaffolds with osteogenic induction components	Both macrophage subtypes inhibit the osteogenic differentiation of ADMSCs on 3D PLGA/PCL scaffolds	Mφs inhibit osteogenic-related pathways (BMP & OSM signaling) during ADSC differentiation
Yang C 2019	Wistar rats—BMSCs; RAW 264.7—Mφs	In vitro & in vivo	In vitro: BMSCs undergo osteogenesis under the CM collected from Mφs stimulated by Ti+LiCl In vivo: the air pouch models are injected with Ti+LiCl	In vitro: LiCl promotes M2 polarization, and the better osteogenic differentiation driven by Ti+LiCl-stimulated CM was also observed In vivo: the LiCl group has fewer infiltrating cells, and thinner fibrous layers further induce higher levels of anti-inflammatory cytokines from M2	LiCl attenuated wear Ti particle-induced inflammation via the suppression of ERK and p38 phosphorylation
Zhu K 2019	C57BL/6 mice—BMSCs; RAW 264.7—Mφs	In vitro & in vivo	In vitro: crocin-pretreated Mφs indirectly cocultured with BMSCs In vivo: the air pouch model is treated with Ti particles+crocin	In vitro: crocin-pretreated Mφs provide an immunomodulatory microenvironment that further promotes osteogenic differentiation In vivo: crocin inhibits Ti particle-induced inflammation and induces M2 polarization	M2 polarization promoted by crocin via the inhibition of p38 and c-Jun N-terminal kinase
Lin T 2019	Balb/c mice—BMSCs; Balb/c mice—Mφs	In vitro	Coculture of BMSCs (preconditioned or genetically modified IL-4-secreting BMSCs) and Mφs directly under the osteogenic medium, including LPS-contaminated polyethylene particles	Both IL-4-secreting BMSCs and preconditioned BMSCs enhance osteogenesis during coculture but at different stages (preconditioned MSCs on day 3 and IL-4-secreting MSCs on day 7)	Enhanced osteogenesis at a later stage associated with the M1-to-M2 Mφ transition

Table 3: Continued.

Author	Cell source	Study type	Cell management	Involvement of Mφ polarization in MSC osteogenic induction	Proposed mechanisms
Wang C 2019	NZW rabbits—BMSCs; RAW 264.7—Mφs	In vitro	Osteogenic differentiation of BMSCs with the supernatants of CS- and Sr-CS-pretreated Mφs	Extracts from Mφs cultured in Sr-CS promote Mφ polarization and enhance BMSC osteogenesis	/
Wendler S 2019	C57BL/6 mice—BMSCs; C57BL/6 mice—Mφs	In vitro & in vivo	In vitro: osteogenic differentiation of BMSCs treated with the CM from bone marrow cells and iloprost In vivo: implantation of a biphasic fibrin scaffold with iloprost into the bone defect	Iloprost decreases the proinflammatory phase and enhances the anti-inflammatory phase to improve bone healing In vivo: postsurgery of receiving iloprost shows an improved fracture healing outcome of the mice	Iloprost signaling leads to an increase of anti-inflammatory agent cAMP to suppress M1
Wu RX 2019	SD rats—BMSCs; SD rats—Mφs	In vivo	Rat periodontal defects are implanted with ECM particles and gels	Gel-type bone ECM has a greater tendency toward M2 polarization showing a better healing tendency	/
Gao A 2020	Human—BMSCs; THP-1—Mφs	In vitro & in vivo	In vitro: BMSCs undergo osteogenic differentiation with Mφ CM collected from the PEEK culture system (rinsing in pH 1.8) In vivo: PEEK (rinsing in pH 1.8) is implanted in the bone defect on the rat femur	In vitro: Mφs in contact with PEEK expressing the M2 phenotype create a more favorable microenvironment for osteogenic differentiation of BMSCs In vivo: the quality and quantity of newly formed bone surrounding the pH 1.8 implants better than the PEEK and O_2 groups	PI3K-Akt signaling, TLR signaling, NLR signaling, and TNF-α signaling all are the mechanisms that alleviate the acute inflammatory response and indirectly enhance osteogenesis

BMSCs: bone marrow stem cells; Mφs: macrophages; LPS: lipopolysaccharides; TGF-β: transforming growth factor-beta; VEGF: vascular endothelial growth factor; IGF-1: insulin-like growth factor 1; CM: conditioned medium; BMP: bone morphogenetic protein; ADSCs: adipose-derived stem cells; IFN-γ: interferon-gamma; OSM: oncostatin M; NT: nanotube; Ti: titanium; sRANKL: soluble receptor activator of nuclear factor-kappa B ligand; OPG: osteoprotegerin; M-CSF: macrophage colony-stimulating factor; NF-κB: nuclear factor-kappa B; HIMC: hierarchical intrafibrillar mineralized collagen; CaPs: calcium phosphates; CDHA: calcium-deficient hydroxyapatite; PLGA/PCL: poly(lactic-co-glycolic) acid/polycaprolactone; Ti+LiCl: titanium+lithium chloride; Sr-CS: strontium-incorporated calcium silicate; cAMP: cyclic adenosine monophosphate; ECM: extracellular matrix; PEEK: polyetheretherketone; PI3K-Akt: phosphoinositide 3-kinase/protein kinase B; TLR: toll-like receptor; NLR: NOD-like receptor; TNF-α: tumor necrosis factor-alpha.

STAT3 and beta-catenin. Both calcium formation and ALP activity of MSCs were decreased when IL-23 in macrophage CM was neutralized by the IL-23 p19 antibody [40].

The effects of M1-to-M2 transition and the persistent proinflammatory status in bone healing have attracted extensive attention. Previous studies have shown that the injury-induced immune response at the proinflammatory stage is necessary for repair progress [32]. 1,25(OH)2D treatment during the inflammatory stage impeded fracture repair and suppressed M1 macrophages while promoting M2 macrophages. The M1-to-M2 transition caused by 1,25(OH)2D was accompanied by decreased release of osteogenic proteins such as OSM, TNF-α, and IL-6 from M1 macrophages. Overall, M1 macrophages are necessary and indispensable for the initiation of the proinflammatory phase during fracture repair [41]. The process of M1-to-M2 transition in a femur defect with LL-37-loaded SFNP Ti implants was demonstrated in He et al.'s study as well. The proinflammatory response of macrophages was largely induced in the injured site on day 4, but M1 macrophages began to decrease on day 7 gradually. The lower M1/M2 ratio after day 7 implies that the M1-to-M2 transition is necessary to improve osteointegration. Peptide LL-37 is more inclined to activate the M1 macrophages but is also capable of inducing anti-inflammatory responses in synergy with the microenvironment and other cytokines [29].

The precise timing of the M1-to-M2 transition for bone formation has been emphasized in the following study. Nathan et al. first utilized LPS-induced M1 macrophages to coculture with MSCs. IL-4 was then added for different durations to induce M2 phenotypes. The results suggest that a 72- to 96-hour proinflammatory environment is critical for appropriate MSC osteogenesis. Interestingly, the optimal time of the M1-to-M2 transition for MSC osteogenesis is gender-dependent. Such sex-linked difference in MSC osteogenesis might be explained by the different levels of steroid receptor expression, which mediates stem cell proliferation and differentiation [42].

3.3.2. Bone Regeneration Enhanced by M2 Macrophages. Individual subtypes of macrophages lead to unique effects on MSCs. Here, we place greater emphasis on the proosteogenic effect of the M2 subtype, especially without any biomaterial involvement. In Gong et al.'s study, M2 macrophages enhanced osteogenic differentiation of MSCs, whereas M1 macrophages impaired it. Proregenerative cytokines, such as TGF-β, VEGF, and IGF-1, were produced by M2 macrophages, and detrimental inflammatory cytokines, such as IL-6, IL-12, and TNF-α, were produced by M1 macrophages and are the suspected mechanisms for the regulation of osteogenic differentiation [43]. However, in Zhang et al.'s study, M0 and M1 macrophages exclusively stimulate the osteogenic differentiation of MSCs in the early and middle stages via OSM and BMP2. In contrast, M2 macrophages are more beneficial to the mineralization of MSCs, the late stage of osteogenesis, in both the direct and indirect coculture systems [44]. He's team also clearly demonstrated how the macrophage subtypes engage in MSC osteogenesis. (1) M0 macrophages had a remarkable effect on promoting osteogenic differentiation. (2) M1 macrophages supported the proliferation of MSCs, while (3) M2 macrophages facilitated MSC osteogenesis. MSCs incubated with CM from M2 macrophages exhibited an enhanced capacity to form robust stem cell sheets [45]. Macrophages converted toward the M2 type by cytokine-preconditioned MSCs and IL-4-secreting MSCs were mentioned in Section 3.2 [28, 34]. Although both preconditioned MSCs and IL-4-secreting MSCs enhanced osteogenesis, there was a significant effect of timing in bone regeneration in vitro. After coculturing with macrophages, preconditioned MSCs promoted bone regeneration at an early stage (day 3), while IL-4-secreting MSC benefits occurred at a later stage (day 7). IL-4-secreting MSCs also possessed greater immunomodulatory capacity on M1-to-M2 transition based on the secretion of IL-4 and PGE2 [46].

3.3.3. Bone Regeneration Enhanced by M2 Macrophages Collaborating with Biomaterials. Bone grafting with an implanted device is a general and promising surgical procedure when bone loss or a fracture has occurred. Besides providing structural stability to the injured site, bone substitutes further benefit osseointegration to its biocompatibility. However, increasing reports indicate that foreign implantation creates an inflammatory environment and forms fibrous capsules leading to negative effects on regeneration. To avoid the dilemma caused by the host-to-scaffold immune response, researchers optimize and improve the scaffolds using various strategies ameliorating the inflammatory environment to enhance the healing.

This section starts with macrophage subtypes triggered by physical factors directly and then addresses the indirect impact of the immune environment. Modifications of the surface properties are commonly being targeted to improve the performances of biomaterials [47, 48]. In Chen et al.'s study, the pore size of the nanoporous anodic alumina was the determinant of macrophage polarization. Compared with the polished material, the nanoporous structures inhibited the expression of proinflammatory cytokines and ROS and induced the shift toward an M2 phenotype. The porous alumina structure stimulated M2 macrophages to express a higher level of osteogenic-inducing factors (BMP2, BMP6, and WNT10b) and fibrosis-enhancing factors (TGF-β1 and VEGF), which are involved in the MSC osteogenesis [49]. Titanium (Ti) metal is widely used in clinical practice due to its remarkable osseointegration capacity. In the following two studies, the different nanostructured surface topographies on Ti that promote macrophage polarization are described. Wang et al. used different Ti specimens, including polished ones (P), ones with nanotubes (NTs) in small diameters (NT-30), and ones with NTs in large diameters (NT-100) to create a microenvironment for macrophage polarization. NT-100 induced M1 polarization and created a prohealing environment, while NT-30 induced M2 polarization, creating an anti-inflammatory environment.

CM from NT-30-induced M2 macrophages enhanced MSC osteogenic differentiation [50]. Ma et al. fabricated superhydrophilic NT TiO_2 surfaces with tube sizes of 30 and 80 nm via anodization at 5 and 20 V (denoted as NT5 and NT20, respectively). Macrophages cultured on NT5 and NT20 surfaces possessed different inflammatory behaviors. The M1 phenotype presented on NT20, whereas the M2 phenotype presented on NT5. NT surface topography and the respective CM acted together to promote the osteogenic behavior of MSCs in vitro. However, NT20-CM increased collagen synthesis and ECM mineralization of MSCs more than NT5-CM. In vivo, NT5 and NT20 both enhanced bone formation after 12 weeks postimplantation [51].

To mitigate the inflammation caused by the implanted materials, anti-inflammatory substances or drugs were applied together with the implanted scaffolds that locally modulated the immune environment. Iloprost, a prostacyclin (PGI2) analog with potent anti-inflammatory properties, was used in bone defects accompanied by a biphasic fibrin scaffold. Wendler's team found that iloprost leads to an increase of anti-inflammatory cAMP that suppresses M1 macrophages. The partial downregulation of inflammation improved bone regeneration outcomes of the mice [52]. The benefits of anti-inflammatory and proregenerative mediators and subsequent increases in M2 macrophages are mentioned in Zhu et al.'s and Yang et al.'s studies. Macrophages were first pretreated with Ti and crocin, an antioxidant and anti-inflammatory compound found in saffron, and then cultured with MSCs in the transwell system. Osteogenic differentiation of MSCs was enhanced due to the M2 polarization promoted by crocin. In addition, crocin polarized the M2 macrophages via the inhibition of p38 and c-Jun N-terminal kinase [53]. Lithium chloride (LiCl) was the selected drug to balance the Ti-induced inflammatory response in Yang et al.'s study. LiCl-derived M2 macrophage polarization and increases in anti-inflammatory and bone-related cytokines further promote MSC osteogenesis [54].

Biomaterials possess unique characteristics that contribute to different immunomodulatory properties and are capable of shaping the local environment as well. Hierarchical intrafibrillar mineralized collagen (HIMC) and strontium-incorporated calcium silicate (Sr-CS) were used in scaffolds to enhance bone regeneration by promoting M2 polarization in vitro and in vivo [55, 56]. HIMC facilitated M2 macrophage polarization and IL-4 secretion to promote MSC osteogenesis. In critical-sized mandible defect models, host MSCs were recruited to the HIMC-loaded IL-4 implantation site and promoted bone regeneration within the anti-inflammatory environment built by HIMC [55]. Similar results were found in Wang et al.'s study; extracts from Sr-CS-pretreated macrophages not only suppressed the inflammatory response but also facilitated MSC osteogenesis and chondrogenesis in vitro. Osteochondral regeneration was significantly improved by Sr-CS in vivo [56]. Calcium phosphates (CaPs), a kind of bone graft material, were applied in the LPS-stimulated macrophage system. CaPs reversed the inflammatory condition caused by LPS-stimulated macrophages, evidenced by the dramatically increased anti-inflammatory-related genes. Osteoclastic-related genes also decreased. The microenvironment created after culturing macrophages on CaPs showed more potent osteogenic effects, fostering osteogenic differentiation of both BMSCs and SaOS-2 cells [57]. ECM bioscaffolds elicited contradictory macrophage phenotypes in Wu et al.'s study. ECM particles had a greater tendency to induce macrophages toward M1 polarization, while ECM gels were more inclined to promote M2 polarization. Although surgical transplantation of ECM particles and ECM gels both showed a better healing tendency in periodontal wounds compared with the control group, the ECM gels showed notable improvements which were attributed to M2 polarization. Notch, PI3K/Akt, integrin, and MEK/ERK are possible signaling pathways responding to the various ECM hydrogels to influence macrophage polarization [58]. Gao et al. performed whole-genome expression analysis to create a map of macrophages that are regulated by biomaterials. Functionalized polyetheretherketone (PEEK) surfaces not only inhibited early proinflammatory M1 polarization but also facilitated M2 differentiation. MSC osteogenesis was promoted after being cultured with the macrophage CM collected from the PEEK surfaces. Inhibited osteoclastogenesis was evidenced by decreased TRAP activity in the macrophages cultured on PEEK surfaces. Thus, enhanced osteogenesis and suppressed osteoclastogenesis synergistically facilitated peri-implant osseointegration. The whole-genome expression analysis of the macrophages was performed after culturing on PEEK for 3 days. The toll-like receptor (TLR), NOD-like receptor (NLR) signaling pathway, and focal adhesion were downregulated, eventually assembling into downstream MAPK and NF-κB signaling cascades to bring about reduced transcription of inflammation-related genes (NOS2, COX2, MIP-1α/β, and CSF1/2). TNF-α and JAK-STAT signaling pathways were also inhibited. Consequently, the autocrine response of macrophages led to an attenuating feedback loop that mitigated the acute inflammatory reaction [59].

3.3.4. Bone Regeneration Inhibited by Macrophages. Although most of the literature shows that macrophages positively benefit MSC osteogenesis, some studies conclude that macrophages inhibit osteogenesis. In Tang et al.'s study, polarized macrophages (M1 or M2) and MSCs formed 3D spheroids at a ratio of 1 to 1 via centrifugation. These 3D spheroids were placed in an osteogenic induction medium for 28 days, and then they examined the degree of osteogenic differentiation. Both subtypes of macrophages inhibited the osteogenic differentiation of MSCs, with M2 macrophages exhibiting an even stronger inhibiting effect than M1 macrophages. N-cadherin was considered the mediator between macrophages and MSCs responsible for the inhibition of osteogenesis [60]. Another study published from the same team followed the same (3D) coculture methods but with poly(lactic-co-glycolic) acid/polycaprolactone scaffolds demonstrating similar results. Downregulated secretion of OSM and bone morphogenetic protein 2 (BMP2) was observed in the

macrophage-MSC cocultures. The gene expression levels of osteogenic markers (ALP, BSP, and RUNX2) were inhibited as well [61]. Multiple factors such as the source of stem cells, polarization strategies for macrophages, and cell ratios are possible explanations for this inhibited osteogenesis. However, the majority of the selected studies in this review support the enhancement of osteogenic differentiation by macrophages. The mechanism behind this phenomenon needs further confirmation and more evidence from rigorous studies.

In summary, macrophages indeed regulate the bone microenvironment to enhance bone healing though the effects of various macrophage subtypes are still under debate. A major proportion of the selected studies demonstrated that M2 macrophages account for the improvement of bone regeneration by both enhancing MSC osteogenesis and repressing inflammation. Biomaterial surface topography could trigger different morphological alterations of macrophages by affecting focal adhesion formation and cytoskeletal structure. The profiles of cytokines released from different subtypes of macrophages promote regeneration at different stages of bone repair. On the other hand, retroregulative cytokines released by stimulated MSCs provide a groundwork for systematically elucidating the likely mechanism and potential targets for enhancing osseointegration. In conclusion, the process and timing of M1-to-M2 transition and its subsequent effects are essential for bone regeneration.

4. Discussion

The field of osteoimmunology started by investigating the effect of the immune system on bone, yet the two decades of osteoimmunology witnessed the emerging role of the skeletal system in the regulation of the immune system, emphasizing the inseparable link between them [62]. The concept of mutual dependency of the two systems must be considered when exploring disease mechanisms or designing therapeutic strategies wherever the skeletal and/or immune systems are involved. Thanks to our improved understanding of osteoimmunology, clinicians can use drugs classically used for osteoporosis to treat immunological (e.g., denosumab for RA). As our understanding progresses and the crosstalk between the two systems is elucidated, they may start looking like a single system [63].

Interaction between MSCs and macrophages has been well established. MSCs have been widely investigated for treating various pathologies with marked inflammation—such as spinal cord injuries—and have shown great anti-inflammatory properties resulting in better outcomes [64]. In vitro and in vivo preclinical studies have shown the essential crosstalk between MSCs and tissue macrophages [65]. Increased understanding of this crosstalk would improve understanding of the immunomodulatory capacity of MSCs and inform the development and testing of potential mechanisms of action to improve therapeutic use of MSCs in treating diseases [66].

While there has already been a review written on the same topic [11], a systematic review has several advantages. By compiling all relevant studies on a particular topic, there is less likely to be biased and we can establish whether findings are consistent and generalizable, which helps clarify current understanding and future directions for readers. Readers can also gauge our review process individually as our protocol is transparent at each phase of the synthesis process [67]. There is a systematic review already published on the effect of MSC secretions on macrophages which is distinct from our systematic review [68]. While we also look at the effect of MSC secretions on macrophages, we further consider the effects of MSCs and macrophages on bone regeneration. As shown in Figure 2, many more papers have been published in the past 3 years about this topic, which shows an increasing relevance and importance in understanding the role of MSCs and macrophages in healing.

MSCs are known to promote polarization of monocytes and macrophages toward the anti-inflammatory (type 2) phenotype and directly inhibit differentiation into the type 1 phenotype and dendritic cells by secreting interleukin-1 receptor antagonist (IL-1RA). Anti-inflammatory monocytes secrete high levels of IL-10, which is crucial for the beneficial effects of MSCs and results in a positive feedback loop of inducing monocyte differentiation toward the anti-inflammatory phenotype [12]. From our systematic review, we found that MSCs induce M2 macrophages, consistent with findings in previous studies. With the increasing relevance of cell therapy, the anti-inflammatory and immunomodulatory nature of MSCs through M2 macrophages makes MSCs an attractive therapeutic option for many diseases [69]. MSC-mediated macrophage polarization has been shown to be beneficial in a myriad of conditions ranging from traumatic spinal cord injury to tendon rupture to dilated cardiomyopathy [70].

Most of our selected studies suggest that M2 macrophages are more important in osteogenesis while M1 macrophages play a minor role. However, some of the selected studies found that M1 macrophages enhanced bone regeneration. These contradictory results can be explained by different subtypes of macrophages exerting unique functions during their respective stages of the healing process. The contribution of M1 and M2 macrophages in fracture healing is sequential and equally important [71]. Classically activated M1 macrophages are inflammatory and further secrete IL-1, IL-6, TNF-α, MCP-1, and MIP-1 to maintain the recruitment of monocytes. They perform phagocytosis to remove necrotic cells as well as the fibrin thrombus formed during healing. Alternatively, activated M2 macrophages are anti-inflammatory and are found more commonly in the later stages of inflammation as they promote tissue repair through IL-10, TGF-beta, BMP2, and VEGF. Their role is to recruit mesenchymal progenitor cells, induce osteochondral differentiation, and prompt angiogenesis.

Despite the proinflammatory effect of M1 macrophages, they are still necessary for the process of healing [5, 8]. In mouse models of acute pancreatitis, depleting macrophages

immediately after the acute inflammatory response significantly reduced duct-like structures. This indicates that M1 macrophages play a key role in acinar-ductal metaplasia which is necessary for healing [72]. Other models also found M1 macrophages critical as depleting macrophages eliminated the benefits of therapeutics that promote M2 differentiation [73]. Although M1 macrophages are necessary for the healing process, their presence over a long period of time was detrimental. Osteoarthritis is associated with an elevated ratio of M1-to-M2 macrophages in peripheral blood. The patients with the higher ratio of M1-to-M2 macrophages in synovial fluid correlated with the more severe osteoarthritis symptom [74].

Classification of M1 or M2 macrophages is normally based on specific markers that tend to be associated with either M1 or M2. M2 macrophages have subclassifications, some of which include markers that have been traditionally considered M1 markers. M2 terminology covers a functionally diverse group of macrophages rather than a uniform activation [75]. Unlike T cells, which undergo extensive epigenetic modifications during differentiation, macrophages retain their plasticity and are responsive to environmental signals. Relying on a single marker to identify a macrophage population can be problematic [76]. Based on this understanding of macrophage classification, we can understand why different studies have different findings regarding the role of M1 and M2 macrophages in promoting MSC osteogenic differentiation. The authors only used a few cell surface markers to classify macrophages, and while it simplifies the process of classification, we find it insufficient in understanding the role of macrophages in bone healing as different macrophages show varying degrees of participation throughout the process.

Among our selected studies, the NF-κB and OSM signaling pathways are most commonly referenced as the mechanisms most likely responsible for the observed interactions between macrophages and MSCs. NF-κB has long been considered a prototypical proinflammatory signaling pathway that regulates multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammatory responses [77]. The proinflammatory cytokines driven by NF-κB are powerful modulators of osteoblast and osteoclast activity. Activation of NF-κB is also crucial for osteoclast differentiation and activation. These characteristics suggest the great potential of NF-κB as a therapeutic target for treating inflammation-associated bone disorders. The effects of NF-κB in osteoblasts are not as clear but have been reported to repress osteoblast differentiation as well as a prosurvival role in osteoblastic cells [78, 79]. Oncostatin M (OSM) belongs to the IL-6 family of cytokines and is associated with multiple biological processes and cellular responses, including growth, differentiation, and inflammation [80]. OSM displays anabolic effects on cortical and trabecular while also driving osteoclast formation. Recruitment of STAT3 or MAPK1/2 by OSM initiated remodeling in conditions like arthritis and osteoporosis and aided in the repair of fractures [81]. OSM stimulates osteoclasts by inducing osteoblastic expression of RANKL, which is mediated by the OSM receptor (OSMR):gp130 receptor complex and downstream initiation of JAK/STAT signaling (namely, STAT3) within osteoblasts [82]. Based on our understanding of this mechanism, macrophage-secreted OSM regulates MSCs and bone cells, which directly impacts the bone remodeling process.

The high regenerative capacity in bone means that most injuries heal well without intervention. Despite this, large defects caused by tumor resections and severe nonunion fractures cannot regenerate properly and require surgery. Currently, the gold standard is autografting but it is limited mainly by its short supply and the morbidity associated with harvesting [83]. Biomaterials are an attractive alternative that can provide the structure necessary for regeneration without the limitations of autografting. These biomaterials were initially "bioinert," but now, many of them are intentionally "bioactive" to augment the healing process. These materials typically consist of bioactive ceramics, bioactive glasses, biologic or synthetic polymers, or composites of the above [84]. However, inflammatory responses occur when these foreign biomaterials are implanted, leading to a cascade of cellular reactions [85]. Neutrophils are responsible for producing inflammatory mediators that promote macrophages differentiating into M1 and M2. If acute inflammation is not resolved, biomaterial-adherent M1 macrophages will begin to form giant cells and transition into chronic inflammation [86]. There is a wide range of treatments to reduce inflammation, but many systemic treatments cannot achieve an adequate local concentration and may have significant adverse effects. Therefore, incorporating anti-inflammatory molecules into solid scaffolds of biomaterials is attractive. Many different molecules capable of reducing inflammation are at various stages of testing. These molecules most commonly target inflammatory cytokines to optimize macrophage polarization [87]. Among the selected studies related to biomaterials, there is substantial evidence that inflammation can be reduced by modulating macrophage polarization. While there are many studies investigating treatments that directly promote healing or affect MSCs to augment healing, we excluded these studies as our systematic review focuses on the relationship between macrophages and MSCs in bone regeneration, and these are not strictly relevant [88–90].

5. Conclusion

The demand for realizing the interaction between MSCs and other cells has soared since transplantation of MSCs is considered a beneficial therapeutic strategy in regenerative medicine. As bone metabolism is tightly regulated by the immune system, macrophages have been drawing attention for their immunomodulatory and osteogenic potential in fracture healing. The crosstalk between MSCs and macrophages during bone regeneration is systematically described in this review. The key points about the crosstalk between these two cells can be roughly divided into two major categories: (1) the effects of transplanted

MSCs on macrophage phenotype switching and (2) how the subtypes of macrophages influence endogenous MSC osteogenesis. MSC transplantation improves bone regeneration and is accompanied by macrophage M2 phenotype switching. Transplanted MSCs and M2 macrophages together create a proresolving environment by enriching specific anti-inflammatory cytokines and osteogenic-inducing factors. Furthermore, M2 macrophages possess great potential for accelerating bone healing in comparison with M0 and M1 macrophages. This review provides compelling evidence that the crosstalk between MSCs and macrophages enhances their regenerative potential on bone via unique secretomes. The phenotype switching time frame of macrophages orchestrates that the microenvironment is crucial for bone regeneration. This review also highlights spatiotemporal changes in the immune system during bone hemostasis. Comprehensive investigations between MSCs and macrophages can extend to other bone diseases and can be beneficial in the clinical application of MSC- or macrophage-based therapies.

Appendix

A. Mesh Terms and Free Words

Mesh terms:

(1) Mesenchymal Stem Cells

(2) Macrophages

(3) Bone regeneration

Free words:

(1) Stem Cell, Mesenchymal

(2) Stem Cells, Mesenchymal

(3) Mesenchymal Stem Cell

(4) Bone Marrow Mesenchymal Stem Cells

(5) Bone Marrow Stromal Cells

(6) Bone Marrow Stromal Cell

(7) Bone Marrow Stromal Cells, Multipotent

(8) Multipotent Bone Marrow Stromal Cells

(9) Adipose-Derived Mesenchymal Stem Cells

(10) Adipose Derived Mesenchymal Stem Cells

(11) Mesenchymal Stem Cells, Adipose-Derived

(12) Mesenchymal Stem Cells, Adipose Derived

(13) Adipose-Derived Mesenchymal Stromal Cells

(14) Adipose Derived Mesenchymal Stromal Cells

(15) Adipose Tissue-Derived Mesenchymal Stem Cells

(16) Adipose Tissue Derived Mesenchymal Stem Cells

(17) Adipose Tissue-Derived Mesenchymal Stromal Cells

(18) Adipose Tissue Derived Mesenchymal Stromal Cells

(19) Mesenchymal Stromal Cells

(20) Mesenchymal Stromal Cell

(21) Stromal Cell, Mesenchymal

(22) Stromal Cells, Mesenchymal

(23) Multipotent Mesenchymal Stromal Cells

(24) Mesenchymal Stromal Cells, Multipotent

(25) Mesenchymal Progenitor Cell

(26) Mesenchymal Progenitor Cells

(27) Progenitor Cell, Mesenchymal

(28) Progenitor Cells, Mesenchymal

(29) Wharton Jelly Cells

(30) Wharton's Jelly Cells

(31) Wharton's Jelly Cell

(32) Whartons Jelly Cells

(33) Bone Marrow Stromal Stem Cells

(34) Bone Marrow-Derived Macrophages

(35) Bone Marrow Derived Macrophages

(36) Bone Marrow-Derived Macrophage

(37) Macrophage, Bone Marrow-Derived

(38) Macrophages, Bone Marrow-Derived

(39) Monocyte-Derived Macrophages

(40) Monocyte Derived Macrophages

(41) Macrophage

(42) Macrophages, Monocyte-Derived

(43) Macrophage, Monocyte-Derived

(44) Macrophages, Monocyte Derived

(45) Monocyte-Derived Macrophage

(46) Bone Regenerations

(47) Regeneration, Bone

(48) Regenerations, Bone

(49) Osteoconduction

B. Recent Queries in PubMed: Search, Query, and Items Found

Please find Figure 3 below for the searching record in PubMed.

2020/1/19 Advanced search - PubMed - NCBI

Search	Add to builder	Query	Items found
#28	Add	Search ((((((((((((((((((((((((((((((((Stem Cell, Mesenchymal[Title/Abstract]) OR Stem Cells, Mesenchymal[Title/Abstract]) OR Mesenchymal Stem Cell[Title/Abstract]) OR Bone Marrow Mesenchymal Stem Cells[Title/Abstract]) OR Bone Marrow Stromal Cells[Title/Abstract]) OR Bone Marrow Stromal Cell[Title/Abstract]) OR Bone Marrow Stromal Cells, Multipotent[Title/Abstract]) OR Multipotent Bone Marrow Stromal Cells[Title/Abstract]) OR Adipose-Derived Mesenchymal Stem Cells[Title/Abstract]) OR Adipose Derived Mesenchymal Stem Cells[Title/Abstract]) OR Mesenchymal Stem Cells, Adipose-Derived[Title/Abstract]) OR Mesenchymal Stem Cells, Adipose Derived[Title/Abstract]) OR Adipose-Derived Mesenchymal Stromal Cells[Title/Abstract]) OR Adipose Derived Mesenchymal Stromal Cells[Title/Abstract]) OR Adipose Tissue-Derived Mesenchymal Stem Cells[Title/Abstract]) OR Adipose Tissue Derived Mesenchymal Stem Cells[Title/Abstract]) OR Adipose Tissue-Derived Mesenchymal Stromal Cells[Title/Abstract]) OR Adipose Tissue Derived Mesenchymal Stromal Cells[Title/Abstract]) OR Mesenchymal Stromal Cells[Title/Abstract]) OR Mesenchymal Stromal Cell[Title/Abstract]) OR Stromal Cell, Mesenchymal[Title/Abstract]) OR Stromal Cells, Mesenchymal[Title/Abstract]) OR Multipotent Mesenchymal Stromal Cells[Title/Abstract]) OR Mesenchymal Stromal Cells, Multipotent[Title/Abstract]) OR Mesenchymal Progenitor Cell[Title/Abstract]) OR Mesenchymal Progenitor Cells[Title/Abstract]) OR Progenitor Cell, Mesenchymal[Title/Abstract]) OR Progenitor Cells, Mesenchymal[Title/Abstract]) OR Wharton Jelly Cells[Title/Abstract]) OR Wharton's Jelly Cells[Title/Abstract]) OR Wharton's Jelly Cell[Title/Abstract]) OR Whartons Jelly Cells[Title/Abstract]) OR Bone Marrow Stromal Stem Cells[Title/Abstract])) OR "Mesenchymal Stem Cells"[Mesh])) AND ((((((((((((Bone Marrow-Derived Macrophages[Title/Abstract]) OR Bone Marrow Derived Macrophages[Title/Abstract]) OR Bone Marrow-Derived Macrophage[Title/Abstract]) OR Macrophage, Bone Marrow-Derived[Title/Abstract]) OR Macrophages, Bone Marrow-Derived[Title/Abstract]) OR Monocyte-Derived Macrophages[Title/Abstract]) OR Monocyte Derived Macrophages[Title/Abstract]) OR Macrophage[Title/Abstract]) OR Macrophages, Monocyte-Derived[Title/Abstract]) OR Macrophage, Monocyte-Derived[Title/Abstract]) OR Macrophages, Monocyte Derived[Title/Abstract]) OR Monocyte-Derived Macrophage[Title/Abstract])) OR "Macrophages"[Mesh])) AND (("Bone Regeneration"[Mesh]) OR ((((Bone Regenerations[Title/Abstract]) OR Regeneration, Bone[Title/Abstract]) OR Regenerations, Bone[Title/Abstract]) OR Osteoconduction[Title/Abstract]))	341

Figure 3

C. Embase: Session Results

Please refer to Figure 4 below for the searching record in Embase.

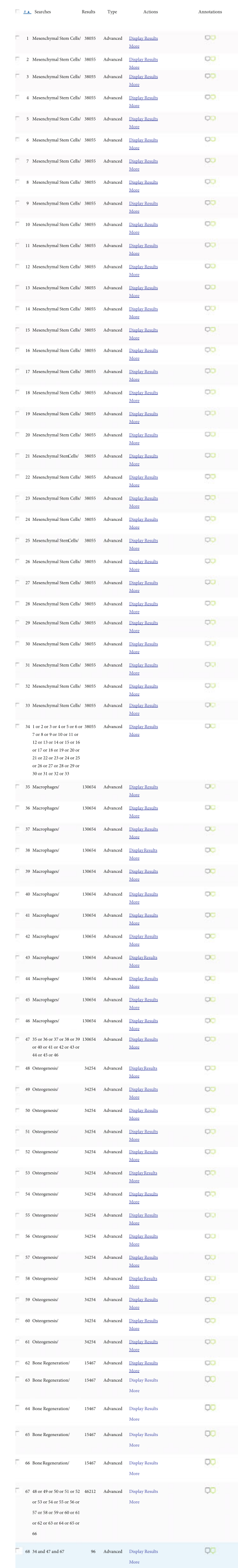

Figure 4

D. Methodological Quality Assessment Document (the Number of "Yes" Answers Was Counted for Each Study to Give a Total Score out of 8)

Please find Tables 4 and 5 below for the quality criteria which are specific to different paragraphs.

E. Summary of Selected Studies and Methodological Score

F. Characteristics of Selected Studies

The induction methods of macrophage phenotypes can be roughly divided into 3 categories: (1) induction by biomaterials, (2) induction by cytokine combination, and (3) induction with gene-modified cells. Refer to the induction by cytokine combination, IFN-γ and LPS were most commonly for M1 induction, and IL-4 was for M2 induction. Flow cytometry analysis and real-time PCR were the most common assessments to pinpoint the subtypes of macrophages. CD11C, CCR7, TNF-α, and CD86 were used to identify M1 macrophages, and CD206, CD36, and CD163 were used to recognize M2 identification in flow cytometry analysis within selected papers. Regarding M1 and M2 marker genes, TNF-α, IL-6, IL-1β, IFN-γ, iNOS, CD86, and OSM represented M1 macrophages, while Arg1, CD206, CD163, IL-10, and Mrc1 were used for M2 macrophages. With respect to measuring the maturation of MSC osteogenesis, osteoblast-related genes, such as *ALP*, *OCN*, *OPN*, *COLI*, *RUNX2*, *IBSP*, and *BMP2*, were detected by real-time PCR and Western blot, and secreted proteins, such as *BMP*, *OSM*, *OPG*, *sRANKL*,

Table 4

Section and topic	No.	Quality criteria	Yes	No
Title/keywords/introduction	1	Were the study hypothesis/aim/objective being clearly described		
	2	Were the experimental design for the study being well described		
	3	Were the method and materials being well described		
Method	4	Were the time points of data collection being clearly defined		
	5	Were the main outcome measurements being clearly defined		
	6	Were the experimental group being well compared with the control group		
Discussion	7	Were the results being well described		
	8	Were the limitation of the article being discussed		

Wells and Littell [22].

Table 5

Study	1	2	3	4	5	6	7	8	Quality score
Tasso et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Seebach et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	8
Tour et al.	Yes	Yes	Yes	Yes	No	Yes	Yes	No	6
Tu et al.	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	8
Gong et al.	Yes	Yes	Yes	Yes	No	No	Yes	No	5
Hirata et al.	Yes	Yes	Yes	Yes	No	Yes	Yes	No	6
Shi et al.	Yes	Yes	Yes	Yes	Yes	No	Yes	No	6
Chen et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Lin et al. (Cytotherapy)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Lin et al. (Stem Cell Res Ther)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Lu et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	8
Saldana et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Tang et al. (Tissue Cell)	Yes	Yes	No	Yes	No	Yes	Yes	No	5
Zhang et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
He et al.	Yes	Yes	Yes	Yes	Yes	Yes	No	No	6
Li et al.	No	Yes	Yes	Yes	Yes	Yes	Yes	No	6
Ma et al.	No	No	Yes	Yes	Yes	Yes	Yes	No	5
Wang et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	8
Wasnik et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	8
Xue et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
He et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Jin et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Lin et al. (Tissue Eng Part A)	No	Yes	Yes	Yes	Yes	Yes	Yes	No	6
Nathan et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	8
Sadowska et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Tang et al. (J Tissue Eng Regen Med)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	8
Wang et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Wei et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Wendler et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Wu et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	8
Yang et al.	Yes	Yes	No	Yes	Yes	Yes	Yes	No	6
Zhu et al.	Yes	No	No	Yes	Yes	Yes	Yes	Yes	6
Gao et al.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7

and *MCSF*, were detected in ELISA. Alizarin Red S staining was used to evaluate calcium deposition/mineralization status during MSC osteogenesis.

Conflicts of Interest

All authors declare that they have no conflict of interest.

Authors' Contributions

Rita Lih-Ying Shin performed the systematic research, analyzed the data, and wrote the manuscript. Chien-Wei Lee edited the manuscript and gave advice on the study. Oscar Yuan-Jie Shen summarized the results and edited the manuscript. Hongtao Xu gave assistance to systematic research. Oscar Kuang-Sheng Lee designed and supervised the study.

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Review Article

Recent Developed Strategies for Enhancing Chondrogenic Differentiation of MSC: Impact on MSC-Based Therapy for Cartilage Regeneration

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Articular cartilage is susceptible to damage, but its self-repair is hindered by its avascular nature. Traditional treatment methods are not able to achieve satisfactory repair effects, and the development of tissue engineering techniques has shed new light on cartilage regeneration. Mesenchymal stem cells (MSCs) are one of the most commonly used seed cells in cartilage tissue engineering. However, MSCs tend to lose their multipotency, and the composition and structure of cartilage-like tissues formed by MSCs are far from those of native cartilage. Thus, there is an urgent need to develop strategies that promote MSC chondrogenic differentiation to give rise to durable and phenotypically correct regenerated cartilage. This review provides an overview of recent advances in enhancement strategies for MSC chondrogenic differentiation, including optimization of bioactive factors, culture conditions, cell type selection, coculture, gene editing, scaffolds, and physical stimulation. This review will aid the further understanding of the MSC chondrogenic differentiation process and enable improvement of MSC-based cartilage tissue engineering.

1. Introduction

Articular cartilage damage is commonly seen in clinical practice and is often caused by trauma, progressive osteoarthritis (OA), and rheumatoid arthritis (RA). Due to its avascular nature, it is difficult for articular cartilage to undergo self-healing [1]. At present, common methods used for articular cartilage regeneration are microfracture [2], particulated articular cartilage implantation [3], osteochondral allograft or autograft transplantation [4, 5], and autologous chondrocyte implantation [6]. However, these techniques are limited in their ability to form hyaline cartilage. The development of cartilage tissue engineering strategies over the past few decades has provided a new approach for cartilage regeneration, which consists of three elements: seed cells, scaffolds, and growth factors [7].

Among various cell types, mesenchymal stem cells (MSCs) are one of the most promising seed cells for cartilage tissue engineering. MSCs are pluripotent adult stem cells that exhibit self-renewal, multipotent differentiation, and immunomodulation functions [8]. The International Society for Cellular Therapy has proposed the following standard criteria for MSCs: (1) MSCs must be plastic adherent in standard culture conditions; (2) MSCs must express CD105, CD73, and CD90 and not express CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR; and (3) MSCs must be able to differentiate into osteoblasts, chondroblasts, and adipocytes *in vitro* [9]. A large number of basic studies and

clinical trials employing MSCs for articular cartilage regeneration have been reported. Intra-articular injection of MSCs has been proven to be safe and effective for improving patients' pain, symptoms, and quality of life [10].

However, MSCs tend to lose their cellular functions, including their self-renewal ability and multipotency, after isolation and *in vitro* expansion, which could in part explain the treatment failures of several MSC-based clinical trials [11]. Numerous studies have indicated that under specific conditions, MSCs can form cartilage-like tissues that contain a certain amount of typical cartilaginous biomolecules, such as type II collagen (COL II), proteoglycans, and aggrecan. However, the composition and structure of the resulting differentiated tissues rarely reach the level of native cartilage. It has been proposed that the collagen content in tissue-engineered cartilage is generally less than 50% of that in native cartilage. In addition, the stratified ultrastructure and spatial organization of native cartilage is often not seen in tissue-engineered cartilage, which results in unsatisfactory mechanical properties [12]. Therefore, differentiating MSCs into normal chondrocytes and maintaining their physiological function are goals that need to be achieved in the field of cartilage regeneration. The regulation of MSC chondrogenic differentiation represents an area that has attracted an enormous amount of research, which is favorable for further understanding of the chondrogenic differentiation process and the optimization of MSC-based cartilage regenerative strategies [13].

In this review, we described the chondrogenic differentiation process of MSCs and then summarized the recent advances in enhancement strategies for MSC chondrogenic differentiation, including optimization of bioactive factors (Table 1), culture conditions, cell type selection, coculture, gene editing, scaffolds, and physical stimulation (Table 2). This review will help to improve the therapeutic effect of MSC-based therapy for cartilage regeneration.

2. Chondrogenic Differentiation Process of MSCs

The cartilage is a connective tissue that is composed of chondrocytes and their surrounding matrix, which mainly contains collagens and proteoglycans. Chondrogenesis, the formation of chondrocytes and cartilage tissues, leads to the development of the various types of cartilage, including hyaline, fibrous, and elastic cartilages [14]. MSCs possess multipotent differentiation potential and can differentiate into numerous mesodermal cell types, such as chondrocytes, osteoblasts, adipocytes, and myofibroblasts [15]. In the process of chondrogenic differentiation, MSCs are thought to follow an endochondral ossification procedure, which includes five main stages (Figure 1). First, in the presence of certain paracrine factors, MSCs produce extracellular matrix (ECM) containing hyaluronan, collagen type I (COL I), and COL II and then undergo increased condensation through cell-ECM and cell-cell interactions. Second, MSCs differentiate into chondrocytes under the influence of a branch of transcription factors, such as Smads, p38, RhoA/ROCK, and SOX9. Third, differentiated chondrocytes proliferate rapidly and secrete ECM. Fourth, mature chondrocytes take on a hypertrophic phenotype and begin to express collagen type X (COL X) and alkaline phosphatase. Fifth, hypertrophic chondrocytes are replaced with blood vessels after cell death [14].

The differentiation of MSCs into chondrocytes requires a dynamic balance of various promoters and inhibitors. The microenvironment consists of soluble cytokines, surrounding matrix, nearby cells, and physical stimuli, all of which play an important role in determining the cellular fates and chondrogenic differentiation of MSCs (Figure 2). However, after differentiating into mature chondrocytes, MSCs may undergo cellular hypertrophy followed by vascular penetration, marrow deposition, and ossification. Exploring potential methods to inhibit unexpected chondrocyte hypertrophy and osteogenic differentiation could help to maintain the phenotype of mature chondrocytes differentiated from MSCs.

3. Bioactive Factors

3.1. Cytokines. Among the multiple cytokines required for initiating MSC chondrogenic differentiation, transforming growth factor beta (TGF-β) is the most commonly used [16]. TGF-β exists in three isoforms, TGF-β1, TGF-β2, and TGF-β3, and has two receptors, TGF-β receptors I (TGF-β RI) and II (TGF-β RII). After binding with TGF-β RI or TGF-β RII, TGF-β induces MSC chondrogenic differentiation mainly through the activation of the TGF-β/Smad signaling pathway. Phosphorylated Smad2/3 binds to Smad4 and translocates into the nucleus, resulting in the expression of *SOX9* and *COL II* [17]. Xu et al. indicated that the activation of RhoA/ROCK was also involved in TGF-β-induced chondrogenic differentiation of rat synovium-derived MSCs (SDSCs) through interaction with the Smad pathway [18]. MAPK signaling is another pathway through which TGF-β regulates MSC chondrogenic differentiation, and in this pathway, p38 promotes chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells (BMSCs), while ERK-1 suppresses BMSC chondrogenic differentiation [19]. Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and also participate in regulating human BMSC (hBMSC) chondrogenic differentiation. Among BMPs, BMP2, BMP4, BMP6, and BMP7 are the most widely employed for BMSC chondrogenic differentiation [20, 21].

In addition to TGF-β and BMPs, other cytokines have also been shown to enhance MSC chondrogenic differentiation. For example, Hagmann et al. revealed that the addition of fibroblast growth factor-2 (FGF-2) during the *in vitro* expansion of hBMSCs significantly enhanced their chondrogenic differentiation with no influence on their adipogenic or osteogenic differentiation [22]. Jeong et al. found that thrombospondin-2 not only promoted the chondrogenic differentiation of the human umbilical cord blood-derived mesenchymal stem cells (UCBSCs) through the activation of the Notch signaling pathway but also attenuated their hypertrophic differentiation [23].

3.2. KGN. Although cytokines play vital roles in inducing MSC chondrogenic differentiation, their applications may

Table 1: Effects of different bioactive factors on MSC chondrogenic differentiation.

Bioactive factors	Cell type	Signaling pathway	Dose	Effect	Ref.
TGF-β3	Human BMSCs	Activate TGF-β/Smad pathway	10 ng/mL	Promote MSC chondrogenic differentiation	[16, 17]
TGF-β1	Rat SDSCs	Activate RhoA/ROCK pathway and Smad pathway	10 ng/mL	Induce gene expression of *SOX9*, *COL I*, *COL II*, and *ACAN*	[18]
	Human BMSCs	Activate MAPK pathway and Wnt pathway	10 ng/mL	Induce gene expression of *SOX9*, *COL II*, and *ACAN* and proteoglycan synthesis	[19]
BMPs	Human ADSCs and BMSCs	NA	500 ng/mL	BMP-2, BMP-4, BMP-6, and BMP-7 are effective enhancers of MSC chondrogenic differentiation	[21]
FGF-2	Human BMSCs	NA	10 ng/mL	Increase GAG/DNA content	[22]
TSP-2	Human UCBSCs	Activate Notch pathway	NA	Promote the chondrogenic differentiation of MSCs and attenuate their hypertrophic differentiation	[23]
KGN	Human ADSCs	NA	100 nM/L	Promote chondrogenic differentiation and suppress chondrocyte hypertrophy in MSCs	[30]
	Human SDSCs	Activate BMP-7/Smad5 pathway	1 μM/L and 10 μM/L	Increase gene expression of *COL II* and *ACAN*	[32]
	Human UCMSCs	Activate JNK/RUNX1 pathway and suppress β-catenin/RUNX2 pathway	1 μM/L	Elevate accumulation of extracellular matrix and chondrogenic gene expression of *SOX9*, *COL II*, and *ACAN*	[33]
	Human BMSCs	NA	100 nM/L	Increase gene expression of *SOX9*, *RUNX2*, *SERPINB2*, and *SERPINA9*	[34]
Melatonin	Human BMSCs	Attenuate IL-1β-induced activation of NF-κB pathway	50 nM/L	Save IL-1β-impaired MSC chondrogenic differentiation	[41]
	Human BMSCs	NA	50 nM/L	Enhance accumulation of GAG, COL II, and COL X	[42]
CS	Goat BMSCs	NA	CS-based hydrogels	Promote MSC chondrogenic differentiation and inhibit chondrocyte hypertrophy	[45]
Ghrelin	Rat BMSCs	Enhance phosphorylation of ERK1/2 and DMNT3A	10 nM/L	Upregulate expression of *COL II*, *SOX9*, and *ACAN* and enhance accumulation of collagen and GAG *in vitro*; improve cartilage repair effect of BMSCs *in vivo*	[50]
Atractylenolides	Rat BMSCs	Activate SHH pathway	30 μg/mL	Increase gene expression of *SOX9*, *COL II*, and *ACAN*	[51]
FSTL-1	Mouse MSCs	Activate TGF-β pathway	5 μg/mL	Upregulate expression of *SOX9* and *COL II*	[53]

TGF-β: transforming growth factor beta; BMSCs: bone marrow-derived mesenchymal stem cells; MSCs: mesenchymal stem cells; SDSCs: synovial membrane-derived mesenchymal stem cells; BMPs: bone morphogenetic proteins; ADSCs: adipose tissue-derived mesenchymal stem cells; NA: not applicable; FGF-2: fibroblast growth factor-2; GAG: glycosaminoglycan; TSP-2: thrombospondin-2; UCBSCs: umbilical cord blood-derived mesenchymal stem cells: KGN: kartogenin; UCMSCs: umbilical cord-derived mesenchymal stem cells; COL II: type II collagen; COL X: type X collagen; CS: chondroitin sulfate; FSTL-1: follistatin-like protein-1.

be restricted due to their short half-life and high cost. Recently, some small molecules have been found to enhance MSC chondrogenic differentiation, and these molecules are particularly intriguing because of their stability and low cost [24]. Kartogenin (KGN), first discovered by Johnson in 2012 [25], is an important small molecule that facilitates MSC chondrogenic differentiation and has drawn considerable interest in recent years [26–28]. Compared with TGF-β, KGN seems to induce a weaker promotion of chondrogenic differentiation but a greater suppression of chondrocyte hypertrophy in human adipose tissue-derived MSCs (ADSCs) [29, 30]. In addition, the combination of KGN and TGF-β3 has synergistic effects, as human umbilical cord-derived mesenchymal stem cells (UCMSCs) treated with KGN and TGF-β3 were shown to secrete more COL II than MSCs treated with TGF-β3 or KGN alone [31]. Zhou et al. reported that KGN can induce the differentiation of human SDSCs (hSDSCs) into chondrocytes through the activation of the BMP-7/Smad5 signaling pathway [32]. In addition, Jing et al. revealed that human UCMSCs (hUCMSCs) preconditioned with KGN were stalled in a precartilaginous stage with the activation of JNK/RUNX1 pathway and suppression of β-catenin/RUNX2 pathway [33]. After induction of chondrogenic differentiation by KGN, hBMSCs expressed significantly increased expression levels of *SERPINA9* and *SERPINB2*, which may serve as novel differentiation markers for MSC lineage commitment toward cartilage [34]. Several biomaterials have been synthesized to improve MSC

Table 2: Effects of different physical stimulation on MSC chondrogenic differentiation.

Physical stimuli	Cell type	Mechanism	Manner	Effect	Ref.
Vibration	Rat BMSCs	Activate Wnt/β-catenin pathway	Low-magnitude (0.49 g) and high-frequency (40 Hz) vibration (30 min/day, 21 days)	Promote MSC chondrogenic differentiation and inhibit hypertrophic differentiation	[123]
Tensile	Rabbit BMSCs	NA	Cyclic dynamic square wave tensile at 5, 10, 15, and 20% of strain, 0.5 Hz (4 h/day, 10 days)	Improve chondrogenic phenotype of MSCs	[124]
Compression	Human BMSCs	Activate TGF-β/Activin/nodal pathway and suppress BMP/GDP and integrin/FAK/ERK pathways	Cyclic dynamic compression force at 5% of strain, 1 Hz (2 h/day, 21days)	Enhance MSC chondrogenic differentiation and suppress chondrocyte hypertrophy	[125]
	Rabbit BMSCs	NA	Cyclic dynamic compression force at 10% of Strain, 1 Hz (2 h/day, 21 days)	Enhance MSC chondrogenic differentiation and suppress chondrocyte hypertrophy and fibrocartilage formation	[126]
Microgravity	Rabbit BMSCs	Suppress IHH and SHH pathways	Rotation at 12–14 rpm for 21 days	Enhance chondrogenic differentiation and attenuate chondrocyte hypertrophy and aging of MSCs	[28]
LIPUS	C3H10T1/2 cells	NA	LIPUS at 30 mW/cm^2, 1 MHz with a pulse duration of 200 μs repeated at 100 Hz (20 min/day)	Increase the expression of *COL II* and *SOX9*	[127]
	Rat BMSCs	Inhibit cell autophagy	LIPUS at 50 mW/cm^2, on–off ratio of 20%, and irradiated with 3 MHz for 20 min (once a day, 10 days)	Increase cartilage-like ECM accumulation and gene expression of *COL II*, *SOX9*, and *ACAN*	[128]
	Rabbit BMSCs	NA	MSC-seeded PGA scaffold was subcutaneously implanted into mouse and treated with LIPUS at 200 mW/cm^2, 0.8 Hz (10 min/day, 4 weeks)	Increase collagen and GAG content and mechanical properties of the scaffold	[129]
Electric field	Human ADSCs	NA	Electric field at 20 mv/cm, 1 kHz (20 min/day, 7 days)	Increase gene expression of *COL II* and *SOX9*; decrease gene expression of *COL I* and *COL X*	[131]
	Mouse BMSCs	Activate $P2X_4$, TGF-β, and BMP pathways	Electrical field at 5 V/cm, 5.0 Hz with a duration of 8 ms for 3 days	Increase gene expression of *COL II*, *SOX9* and *ACAN* and accumulation of COL II and GAG	[133]
	Swine BMSCs	Downregulate the expression of DMMT1 and increase methylation of the promoters of OCT4 and NANOG	Nanosecond pulsed electrical field of 10 ns at 20 kV/cm or 100 ns at 10 kV/cm, 1 Hz for 14 days	Enhance cartilaginous ECM accumulation and gene expression of *COL II* and *SOX9*	[135]
Electromagnetic field	Human BMSCs	NA	Electromagnetic field at 5 mT, 15 Hz (45 min/8 h, 21 days)	Increase gene expression of *COL II* and GAG/DNA content	[137]
	Human BMSCs	Stimulate calcium influx	Electromagnetic field at 2 mT, 15 Hz for 10 min once on day 1 induction	Enhance cartilaginous ECM deposition and gene expression of *COL II* and *SOX9*	[138]

BMSCs: bone marrow-derived mesenchymal stem cells; MSCs: mesenchymal stem cells; NA: not applicable; LIPUS: low-intensity pulsed ultrasound; ECM: extracellular matrix; PGA: polyglycolic acid; ADSCs: adipose tissue-derived mesenchymal stem cells; COL II: type II collagen; GAG: glycosaminoglycan.

chondrogenic differentiation through controlled release of KGN. For example, Sun et al. developed a collagen/chitosan/-hyaluronic acid (HA) scaffold containing poly(lactide-co-glycolide) (PLGA) microspheres for controlled KGN release and cartilage regeneration [35]. Chen et al. fabricated a KGN-conjugated poly(ether-ester-urethane)urea scaffold

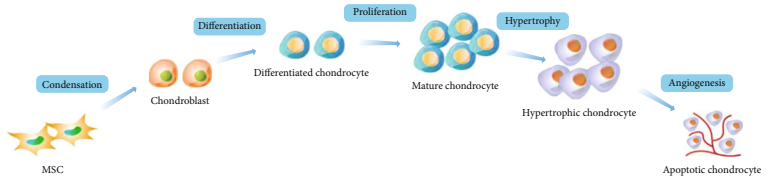

Figure 1: Chondrogenic differentiation process of mesenchymal stem cells (MSCs). The chondrogenic differentiation of MSCs is proposed to follow an endochondral ossification procedure, which includes five main stages: condensation, differentiation, proliferation, hypertrophy, and angiogenesis.

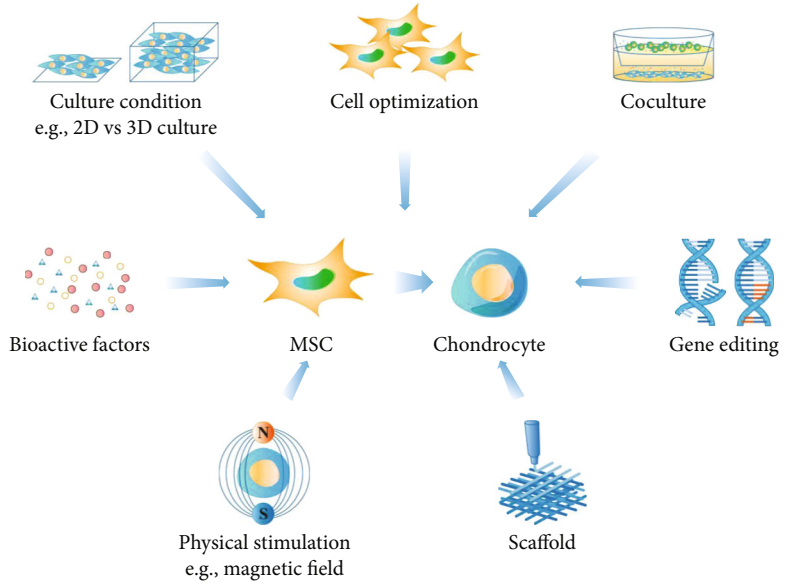

Figure 2: Approaches for enhancing MSC chondrogenic differentiation. Several methods have proven to be effective in promoting chondrogenic differentiation of MSCs, including optimization of bioactive factors, culture conditions, cell type selection, coculture, gene editing, scaffolds, and physical stimulation.

and demonstrated that KGN on the scaffold could undergo stable sustained release, thus enhancing chondrogenic differentiation of hUCMSCs *in vitro* and cartilage regeneration in rabbits [36].

3.3. Melatonin. Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine that was first isolated from the pineal tissue in 1957 [37]. In addition to participating in the modulation of various physiological functions, such as sleep, circadian rhythms, and neuroendocrine processes, recent studies have suggested that melatonin also plays an important role in regulating MSC differentiation [38, 39]. It has been proven that melatonin enhances hBMSC osteogenic and chondrogenic differentiation while inhibiting adipogenic differentiation [40, 41]. Gao et al. performed a study in which they induced hBMSC chondrogenic differentiation with chondrogenic medium containing vehicle or melatonin. They found that the synthesis of glycosaminoglycans (GAGs) and COL II and the gene expression levels of *ACAN*, *COL II*, and *SOX9* were higher in the melatonin group than in the control group. Furthermore, they confirmed that melatonin receptors were expressed on chondrogenic BMSCs. After treatment with a melatonin receptor antagonist, the effect of melatonin on the chondrogenic differentiation of BMSCs was blocked, indicating that melatonin promoted BMSC chondrogenic differentiation at least partially through melatonin receptors [42].

3.4. Chondroitin Sulfate. Chondroitin sulfate (CS), a type of GAG in connective tissues, has shown the capacity to enhance MSC chondrogenic differentiation by providing a chondroinductive microenvironment [43, 44]. Compared with poly(ethylene glycol) hydrogels, CS-based hydrogels are able to promote both chondrocyte-specific gene expression and cartilage ECM accumulation. Furthermore, CS can inhibit the hypertrophic differentiation of goat BMSCs, as evidenced by significantly downregulated expression of *COL X* [45, 46]. The stiffness of the hydrogels also has an impact on the function of CS. CS-containing hydrogels with low mechanical stiffness were reported to lead to more neocartilage deposition than those with high stiffness [47]. CS supplementation has been utilized as a biochemical cue in integrated cartilage tissue engineering. Moura et al. developed 3D porous poly(ε-caprolactone) scaffolds with CS supplementation, which were able to promote hBMSC proliferation, migration, and chondrogenic differentiation [48]. Similarly, Huang et al. fabricated an alginate foam scaffold supplemented with CS and found increased amounts of

a cartilage-specific matrix in differentiated hBMSC cultures supplemented with CS than in those supplemented with CS-free foams [49].

3.5. Other Factors. In addition to the above bioactive factors, other factors modulating MSC chondrogenic differentiation have also been investigated. Fan et al. demonstrated that ghrelin, also called the "hunger hormone," significantly promoted rat BMSC chondrogenic differentiation, as evidenced by the upregulated expression of *COL II*, *SOX9*, and *ACAN* and enhanced accumulation of collagen and GAGs *in vitro*, which may be related to increased intracellular phosphorylation of ERK1/2 and DNMT3A. Furthermore, delivery of ghrelin and TGF-β3 significantly improved the cartilage repair effect of BMSCs in rats compared with delivery of TGF-β3 alone [50]. In addition, Li et al. reported that atractylenolides, a traditional Chinese medicine, was able to promote rat BMSC chondrogenic differentiation via activation of the Sonic Hedgehog (SHH) signaling pathway [51]. Follistatin-like protein-1 (FSTL-1), an acidic cysteine-rich glycoprotein, also plays a role in regulating MSC chondrogenic differentiation [52]. FSTL-1-deficient mouse embryonic skull-derived MSCs exhibited significantly downregulated gene expression of *COL2A1* and *SOX9*, reduced ECM production, and decreased activity of the TGF-β signaling pathway [53].

4. Culture Conditions

MSCs tend to lose their differentiation potential as a result of culture stress or cell senescence when expanded *in vitro*. Articular cartilage resides at low oxygen tension (1-4% oxygen) *in vivo* [54]. The impact of hypoxia on MSC chondrogenic differentiation has been of particular interest. It was demonstrated that MSCs cultured under low oxygen tension exhibited enhanced early chondrogenic differentiation and reduced hypertrophic differentiation, as evidenced by higher expression levels of the chondrogenic markers *COL II*, *SOX9*, and *ACAN* and lower expression levels of the hypertrophic markers *COL X* and *MMP13* [55–57]. Portron et al. investigated the related intracellular mechanism and confirmed that low oxygen tension increased the DNA-binding activities of two biological effectors, HIF-1α and HIF-2α, which have been reported to be promoters of human ADSC (hADSC) chondrogenic differentiation [55]. In addition, recent studies of cartilage tissue engineering have investigated the effect of 3D culture on MSC chondrogenic differentiation, which represents a potential way to mimic the *in vivo* cartilage tissue environment. Synthetic and natural materials, such as 3D-printed bioreactor chambers, hydrogels, and microspheres, have been developed as tools to create a 3D microenvironment for MSCs [58–61]. For example, Sulaiman et al. compared the 2D and 3D cultures of hBMSCs and found that 3D culture of BMSCs on gelatin microspheres enhanced their stemness and chondrogenic differentiation compared to 2D culture on a standard tissue culture plate [61].

5. Cell Types

In recent years, many researchers have proposed that MSCs are heterogeneous and that not all share the same chondrogenic differentiation abilities. This heterogeneity was reported to exist among different donors, tissue sources, and cell phenotypes.

Among the various donor characteristics, the effect of donor age on MSC chondrogenic differentiation ability has been most frequently studied. Kanawa et al. isolated BMSCs from 17 patients (25-81 years old) and expanded them with FGF-2 for 28-42 days before differentiation assays. After 28 days of induced culturing, they found that the chondrogenic potential, rather than the osteogenic or adipogenic potential, of BMSCs declines with donor age, as evidenced by decreases in the expression of chondrocyte-specific genes such as *SOX9*, *COL2A*, and *ACAN*. Moreover, the (GAGs)/DNA content also significantly decreased with donor age after chondrogenic differentiation [62]. However, Andrzejewska et al. indicated that the chondrogenic potential of BMSCs was not affected by donor age. They examined the phenotypic and functional performances of BMSCs isolated from adult and elderly patients (n=10 and n=13, mean age 38 and 72 years old) and found no difference in proteoglycan synthesis between BMSCs (at passage 6) from younger adults and those from older adults after 21 days of chondrogenic differentiation induction [63]. Thus, it is still not clear whether MSC chondrogenic differentiation is affected by donor age, and further studies are needed. On the other hand, Dudics et al. demonstrated that the chondrogenic differentiation ability of BMSCs from OA and RA patients was comparable to that of BMSCs from healthy individuals, as shown by similar *COL II* gene expression and proteoglycan synthesis after chondrogenic induction, suggesting that BMSCs from OA and RA patients could also be applied in cartilage tissue engineering [64]. Garcı'a-A'lvarez reached a similar conclusion when they found that the chondrogenic differentiation potential of BMSCs from OA patients was similar to that of BMSCs from femoral fracture patients [65].

Conversely, it is well recognized that MSCs from different tissue sources possess different potentials for chondrogenic differentiation. Compared with BMSCs, ADSCs appear to have lower chondrogenic potential [66–68]. MSCs have also been identified in the synovial tissue, a tissue type that is adjacent to articular cartilage. SDSCs have shown higher chondrogenic potential than BMSCs and ADSCs [69]. However, Neybecker revealed that the chondrogenic differentiation potential of SDSCs was lower than that of BMSCs in advanced OA patients, which may be attributed to the intraarticular inflammatory environment caused by OA [70]. It has also been proposed that the chondrogenic differentiation and ECM production capacities of human amnion- and placenta-derived MSCs are higher than those of hADSCs [71, 72]. The chondrogenic differentiation potential of MSCs derived from the same tissue in different parts of the body also varies. For example, compared with those isolated from the femoral head bone marrow, hBMSCs isolated from the iliac crest and vertebral body bone marrow were more likely to differentiate into chondrocytes and form cartilaginous tissue *in vitro* [73].

MSCs from the same tissue are different in cellular phenotype. $CD105^+$ SDSCs possess greater chondrogenic potential than $CD105^-$ SDSCs. The promotion of SDSC chondrogenic differentiation by CD105 is achieved through the activation of the TGF-β/Smad2 signaling pathway [74–76]. Hagmann et al. revealed that after chondrogenic differentiation, $CD146^+$ hBMSCs produced more GAGs than unsorted BMSCs [77]. Compared with $CD106^+$ or $CD73^+$ hSDSCs, $CD271^+$ SDSCs exhibited a greater chondrogenic differentiation capacity, as determined by histological and immunohistochemical analyses for COL II [78]. Single-cell RNA sequencing (scRNA-seq) technology can be used to analyze gene expression at the single-cell level, enabling the identification of functional cell subpopulations, making it a powerful tool for investigating MSC heterogeneity [79]. Freeman et al. used scRNA-seq to assess the transcriptional diversity of mouse BMSCs and found that the expression of genes associated with multilineage potential and immunomodulation ability was inconsistent between individual cells [80]. Sun et al. investigated the gene expression profile of human Wharton's jelly MSCs (WJMSCs) via scRNA-seq and found some highly variable genes to be associated with the functional properties of WJMSCs. They found that different subpopulations showed distinct chondrogenic differentiation potency [81]. By performing scRNA-seq of the transcriptome, Liu et al. identified 3 subpopulations within hBMSCs, among which one subpopulation exhibited a strong expression of FGFR2 and potentially included skeletal stem cells [82]. Specifically, Merrick et al. demonstrated that dipeptidyl peptidase-4/$CD26^+$ ADSCs represent highly proliferative and multipotent progenitors in murine and human adipose tissues, while their chondrogenic differentiation ability still needs further investigation [83]. Additional research is needed to explore more functional MSC subpopulations via scRNA-seq to identify those with greater chondrogenic differentiation potential.

6. Coculture

Coculture was first performed in 1978 by Lawrence et al., who indicated that heterologous cells communicated and responded to cell-specific hormones through cyclic AMP [84]. In recent years, coculture has been applied in cartilage tissue engineering [85]. It was reported that the presence of chondrocytes promoted MSC chondrogenic differentiation in culture [86, 87]. Compared to direct coculture, indirect coculture with human UCBSCs and chondrocytes significantly increased the expression of *SOX9* and *COL II* and decreased the expression of *COL I* in UCBSCs [88]. Kubosch et al. revealed that coculture of human or swine SDSCs with chondrocytes resulted in greater self-organization, chondrogenic differentiation, and TGF-β secretion in SDSCs, suggesting that chondrocytes may induce a chondrogenic phenotype in SDSCs through paracrine action mimicking joint homeostasis [89, 90]. *In vivo* ectopic chondrogenic differentiation of swine BMSCs could also be induced by mature chondrocytes, which may be attributed to soluble chondrogenic factors secreted by chondrocytes [91]. In addition, when cocultured with hADSCs, chondrocytes were shown to suppress the undesired hypertrophy of hADSCs [92]. Zhang et al. carried out a study in which human WJMSCs and chondrocytes were cocultured on an acellular cartilage ECM scaffold and transplanted into the articular cartilage defect area in caprine. After 9 months, they found that the neotissue was more similar to native cartilage than that formed by the transplantation of WJMSCs or chondrocytes alone, indicating that coculture represents a promising strategy for improving the cartilage-regenerating effects of MSCs [93]. However, to determine the optimal culture conditions, MSC and chondrocyte cocultures need to be further investigated in more *in vivo* models. In addition, the impact of coculturing MSCs with other cell types on MSC chondrogenic differentiation should also be evaluated [94].

7. Gene Editing

The overexpression and knockdown of specific genes are optional methods to control chondrogenic differentiation in MSCs. *DLX5* is a member of the *DLX* gene family, and DLX5 associates with HOXC8 to form a protein complex. Yang et al. revealed that the expression of both *DLX5* and *HOXC8* was increased during chondrogenic differentiation of human apical papillae-derived MSCs (APSCs) and that the overexpression of *DLX5* and *HOXC8* promoted the chondrogenic differentiation of APSCs. In fact, the protein complex formed by *DLX5* and *HOXC8* could inhibit the activation of *LINC01013*, a negative regulator of chondrogenesis, by directly binding to its promoter [95]. Similarly, *KLF15*, a member of the *KFL* transcription factor family, is also upregulated when hBMSCs undergo chondrogenic differentiation. By binding to the *SOX9* promoter, *KFL15* was shown to activate *SOX9* and enhance the chondrogenic differentiation potential of BMSCs [96]. In addition, Zhou et al. found that *corin* expression was upregulated in the trilineage differentiation process of hBMSCs. The silencing of *corin* gene expression inhibited chondrogenic (rather than osteogenic and adipogenic) differentiation of BMSCs, indicating that *corin* may play a positive role in the regulation of chondrogenic differentiation of BMSCs [97]. Tian et al. demonstrated that miR-30a also plays an important role in chondrogenic differentiation of rat BMSCs by inhibiting *DLL4* expression [98]. In another study, Kim et al. fabricated *shATF4* and *SOX9* plasmid DNA complexed with gene regulation nanoparticles and verified that it could significantly promote the chondrogenic differentiation of hBMSCs [99]. In addition, it was demonstrated that *H-89* could increase miR-23b expression in human MSCs (hMSCs), thus promoting their chondrogenic differentiation through inhibition of PKA signaling [100]. All of these genes may be potential targets for gene editing to enhance MSC chondrogenic differentiation. However, the safety of gene editing in MSCs needs to be fully explored before this strategy can be applied clinically.

8. Scaffolds

Researchers are constantly attempting to fabricate scaffolds that are able to enhance MSC chondrogenic differentiation. It has been proposed that the physical properties of the

scaffolds are involved in regulating MSC chondrogenic differentiation. Ahmed et al. developed 16 electrospun scaffolds with different stiffness and wettability and revealed that chondrogenic differentiation of ATDC5 cells were enhanced in soft scaffolds with an intermediate wettability as evidenced by an increased level of cartilage-associated gene expression [101]. In another study, Nalluri et al. synthesized a hydrophilic polyurethane scaffold with gel like architecture and found that it enhanced BMSC chondrogenic differentiation, as determined by significantly increased cartilage-specific ECM production [102]. Additionally, the porosity and pore size of scaffolds also play a role in MSC chondrogenic differentiation. Prasopthum et al. demonstrated that 3D-printed scaffolds with micro/nanoporous structures could promote chondrogenic and osteogenic differentiation of hBMSCs better than scaffolds with nonporous structures [103]. It was reported that small-pore scaffolds (pore size of 125-250 μm) were more likely to enhance chondrogenic differentiation and inhibit endochondral ossification of hBMSCs compared with large-pore scaffolds (pore size of 425-600 μm) [104]. Interestingly, Di Luca et al. created scaffolds composed of poly(ethylene oxide therephtalate)/poly(butylene therephtalate) with a structural gradient in pore size. They confirmed that hBMSCs seeded on the gradient scaffolds produced more GAGs as compared with those seeded on nongradient scaffolds [105].

As a biologically complete substrate, ECM has been proposed to provide a native microenvironment for MSCs and to aid in the maintenance of their functions [106, 107]. Coating with ECM has been shown to preserve the stemness and differentiation potential of *in vitro*-expanded MSCs [108]. Compared with polyglycolic acid (PGA) scaffolds, ECM scaffolds not only enhanced chondrogenic differentiation of rabbit BMSCs more effectively but also maintained the BMSC phenotype for longer *in vivo* [109]. Li et al. demonstrated that cartilage ECM could not only enhance chondrogenic differentiation but also inhibit hypertrophic differentiation of hBMSCs. Among various ECM collagen subtypes, collagen type XI exhibited the strongest effects on promoting the production and inhibiting the degradation of cartilage matrix [110]. Collagen and GAGs are ideal natural materials that can mimic the matrix niche of chondrocytes and reportedly have an enhancing effect on the chondrogenic differentiation of MSCs [111]. Raghothaman et al. fabricated an interfacial polyelectrolyte complexation-Col I hydrogel and found that it could enhance cell-cell interactions and cellular condensation, thereby resulting in improved hBMSC chondrogenic differentiation and hyaline neocartilage formation [112]. In another study, Meng et al. generated a tricalcium phosphate-collagen-hyaluronan scaffold and found that it efficiently induced chondrogenic differentiation of ATDC-5 cells and hBMSCs without the need for exogenous growth factors [113]. Similarly, Moulisová et al. constructed a gelatin-HA hybrid hydrogel and confirmed that it promoted both chondrogenic differentiation and adhesion of hBMSCs [114]. Feng et al. synthesized sulfated HA hydrogels and found that they not only promoted MSC chondrogenic differentiation but also suppressed hMSC hypertrophy. When utilized to treat OA in rats, the sulfated HA hydrogels significantly reduced cartilage abrasion and hypertrophy [115].

Additionally, previous works have shown that biomaterials can be used as effective delivery vehicles or bioactive matrices to promote MSC chondrogenic differentiation and mitigate MSC hypertrophy. Morille et al. generated PLGA-based microspheres coated with TGF-β3 and confirmed their promotion of chondrogenic differentiation of MSCs *in vitro*. When hBMSCs seeded onto these microspheres were injected into the knee cavities of rats with OA, cartilage-like tissue was formed, and decreased degradation of endogenous articular cartilage was observed after 6 weeks [116]. In addition, Xu et al. fabricated a multifunctional nanocarrier modified with RGD peptide and β-cyclodextrin that could carry siRNA targeting Runx2 and small molecules such as KGN. They verified that it was able to induce hMSC differentiation into chondrocytes and suppress their hypertrophy [117]. Remote control of MSC chondrogenic differentiation *in vivo* via biomaterials has also been achieved. Based on an upconversion nanotransducer, Kang et al. developed a nanocomplex with photolabile caging of KGN and calcium, whose release could be triggered by near-infrared light. They confirmed that intracellular KGN and calcium delivery promoted chondrogenic differentiation and inhibited the hypertrophy of hMSCs *in vivo* [118].

9. Physical Stimulation

9.1. Mechanical Stimulation. Articular cartilage is a smooth wear-resistant connective tissue that can withstand complex mechanical stimuli and distribute loads to the subchondral bone. Proper mechanical stimulation has been revealed to upregulate the gene expression of *ACAN* and *COL II* in chondrocytes while maintaining their phenotypes, thus promoting cartilage formation [119–121]. Similarly, an in-depth understanding of the effect of mechanical stimulation on MSC chondrogenic differentiation may facilitate the success of MSC-based cartilage regenerative therapies in joints, which have a mechanically demanding environment. It is proposed that MSCs respond to mechanical stimulation through autocrine or paracrine activity to enhance their chondrogenic differentiation and capacity for repairing cartilage damage. Various types of mechanical stimulation have been applied to enhance MSC chondrogenic differentiation in cartilage tissue engineering [122]. Hou et al. demonstrated that low-magnitude high-frequency vibration enhanced the chondrogenic potential of rat BMSCs through activation of the Wnt/β-catenin signaling pathway [123]. Xie et al. revealed that proper tensile mechanical stimulation could improve the viscoelasticity and chondrogenic phenotype of rabbit BMSCs [124]. Additionally, Zhang et al. investigated the effect of deferral dynamic compression on the chondrogenic differentiation of hBMSCs and found that it enhanced chondrogenic differentiation and suppressed chondrocyte hypertrophy, accompanied by the activation of TGF-β/Activin/Nodal signaling pathway and suppression of BMP/GDP and integrin/FAK/ERK signaling pathways [125]. Cao et al. performed a similar study in which they applied dynamic

mechanical loading to rabbit BMSCs-collagen scaffold constructs and found that BMSCs expressed higher levels of *ACAN*, *COL2A1*, and *SOX9* and lower levels of *COL10A1* and *COL1A2*. The mechanical strength of the constructs was significantly improved and was similar to that of native cartilage [126]. Indian Hedgehog (IHH) and SHH can promote MSC chondrogenic differentiation but tend to result in chondrogenic hypertrophy and ossification. Chen et al. reported that microgravity caused by a rotary cell culture system was able to enhance chondrogenic differentiation of rabbit BMSCs while attenuating the chondrocyte hypertrophy and aging induced by IHH and SHH [28].

In addition, recent studies have demonstrated that low-intensity pulsed ultrasound (LIPUS), which provides mechanical stimulation in the form of sound waves, can be used to promote chondrogenic differentiation of C3H10T1/2 cells [127]. After LIPUS stimulation at 3 MHz, BMSCs secreted increased amounts of cartilage-like ECM and showed upregulated expression of chondrogenic genes, such as *COL II*, *SOX9*, and *ACAN*. The stimulatory effect of LIPUS on rat BMSC chondrogenic differentiation is reportedly achieved through inhibition of autography [128]. Cui et al. seeded rabbit BMSCs on a PGA scaffold and implanted the construct into the backs of nude mice, which subsequently received LIPUS stimulation for 10 min every day for 4 weeks. They found that the collagen and GAG content, as well as the mechanical properties, showed a more significant increase in the LIPUS group than in the unstimulated group, suggesting that LIPUS stimulation could promote BMSC chondrogenic differentiation *in vivo* [129].

9.2. Electric Field. In addition to mechanical stimulation, other physical stimuli, such as electrical and electromagnetic/magnetic stimuli, also have an impact on the chondrogenic differentiation of MSCs [130]. Treatment with a low-frequency electric field (EF) was reported to result in increased expression of *COL II* and *SOX9* and decreased expression of *COL I* and *COL X* in hADSCs [131, 132]. Even in the absence of exogenous growth factors, a low-frequency EF could enhance chondrogenic differentiation of mouse BMSCs. It was demonstrated that EF promoted BMSC chondrogenic differentiation by driving Ca^{2+}/ATP oscillations, which are known to play an important role in prechondrogenic condensation. In addition, EF was found to induce increased TGF-β1 expression, and the inhibition of TGF-β signaling blocked EF-driven BMSC chondrogenic differentiation, indicating that TGF-β signaling mediates EF-driven BMSC chondrogenic differentiation. Other signaling pathways, including BMP signaling and MAPK signaling, have also been proposed to be involved in regulating the effect of EF treatment on BMSC chondrogenic differentiation [133, 134]. Additionally, Li et al. revealed that nanosecond pulsed EF (nsPEF) downregulated the expression of *DMMT1*, thus increasing the methylation of the *OCT4* and *NANOG* promotors. As a result, swine BMSCs treated with nsPEF exhibited enhanced trilineage differentiation ability [135].

9.3. Electromagnetic Field. Electromagnetic field (EMF) has also been shown to promote MSC chondrogenic differentiation [136]. Mayer-Wagner et al. investigated the impact of EMF on hBMSCs during chondrogenic differentiation and found that BMSCs exposed to a low-frequency EMF (5 mT) showed higher *COL II* expression, increased (GAGs)/DNA content, and lower *COL X* expression than those that had not been treated with an EMF [137]. Analogously, Parate et al. demonstrated that optimal hBMSC chondrogenic differentiation was achieved with a brief (10 min), low-intensity (2 mT) pulsed EMF exposure before chondrogenic induction rather than prolonged and repetitive EMF exposure. Transient receptor potential channels, a conduit for extracellular calcium, might be involved in mediating pulse EMF-driven BMSC chondrogenic differentiation [138].

10. Conclusions and Perspectives

MSCs have shown great prospects in cartilage tissue engineering. However, some issues need to be resolved before they can be widely applied. First, MSC-based therapy is largely limited by the ability to obtain and manufacture applicable MSC products because MSCs expanded *in vitro* are prone to losing their therapeutic potential and safety attributes [139]. Developing strategies to enhance chondrogenic differentiation in MSCs is necessary and has important clinical value for cartilage regeneration. In the present review, we summarized the recent research progress in MSC chondrogenic differentiation modulation, including optimization of bioactive factors, culture conditions, cell type selection, coculture, gene editing, scaffolds, and physical stimulation. Although all of these methods are effective in regulating chondrogenic differentiation of MSCs, the reliability, safety, and degree of difficulty in implementing these methods need to be considered. Second, because MSCs tend to undergo hypertrophy in their chondrogenic differentiation process, it is difficult for them to form hyaline cartilage *in vivo* [12]. A more comprehensive understanding of embryonic chondrogenesis would be beneficial for guiding MSCs to differentiate into cells with a cartilage phenotype. It has been suggested that MSC chondrogenic differentiation may occur in two different directions: one leading to bone formation via endochondral ossification and the other leading to articular cartilage formation. Although endochondral ossification has been widely used as a model to establish MSC chondrogenic differentiation protocols, chondrogenic differentiation of cartilage chondrocytes should be used instead to alleviate inevitable hypertrophic differentiation [140]. Third, the underlying mechanisms by which endogenous and transplanted MSC function remain to be elucidated. In-depth research has revealed that MSCs can perform a paracrine action and are capable of secreting diverse bioactive molecules, such as growth factors, cytokines, and chemokines [141, 142]. It is suggested that the chondrogenic differentiation of endogenous MSCs is involved in cartilage regeneration, but this is not necessarily true for implanted MSCs, which mainly work through immunomodulatory functions. To further improve the cartilage-regenerating ability of MSCs, additional strategies to recruit host MSCs and enhance their chondrogenic differentiation are still needed.

It is also essential to exploit approaches to enhance MSC paracrine and immunomodulatory functions.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

KZ designed the study, collected data, and contributed to manuscript writing. ZS collected data and contributed to manuscript writing. YY designed the study and figures. MC analyzed data and provided comments. CG, LF, and HL collected data. XS provided the study material. QG and SL designed the study and contributed to manuscript writing. All authors read and approved the final manuscript. KZ and ZS contributed equally to this work.

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Research Article

Lithium and Copper Induce the Osteogenesis-Angiogenesis Coupling of Bone Marrow Mesenchymal Stem Cells via Crosstalk between Canonical Wnt and HIF-1α Signaling Pathways

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The combination of osteogenesis and angiogenesis dual-delivery trace element-carrying bioactive scaffolds and stem cells is a promising method for bone regeneration and repair. Canonical Wnt and HIF-1α signaling pathways are vital for BMSCs' osteogenic differentiation and secretion of osteogenic factors, respectively. Simultaneously, lithium (Li) and copper (Cu) can activate the canonical Wnt and HIF-1α signaling pathway, respectively. Moreover, emerging evidence has shown that the canonical Wnt and HIF signaling pathways are related to coupling osteogenesis and angiogenesis. However, it is still unclear whether the lithium- and copper-doped bioactive scaffold can induce the coupling of the osteogenesis and angiogenesis in BMSCs and the underlying mechanism. So, we fabricated a lithium- (Li^+-) and copper- (Cu^{2+}-) doped organic/inorganic (Li 2.5-Cu 1.0-HA/Col) scaffold to evaluate the coupling osteogenesis and angiogenesis effects of lithium and copper on BMSCs and further explore its mechanism. We investigated that the sustained release of lithium and copper from the Li 2.5-Cu 1.0-HA/Col scaffold could couple the osteogenesis- and angiogenesis-related factor secretion in BMSCs seeding on it. Moreover, our results showed that 500 μM Li^+ could activate the canonical Wnt signaling pathway and rescue the XAV-939 inhibition on it. In addition, we demonstrated that the 25 μM Cu^{2+} was similar to 1% oxygen environment in terms of the effectiveness of activating the HIF-1α signaling pathway. More importantly, the combination stimuli of Li^+ and Cu^{2+} could couple the osteogenesis and angiogenesis process and further upregulate the osteogenesis- and angiogenesis-related gene expression via crosstalk between the canonical Wnt and HIF-1α signaling pathway. In conclusion, this study revealed that lithium and copper could crosstalk between the canonical Wnt and HIF-1α signaling pathways to couple the osteogenesis and angiogenesis in BMSCs when they are sustainably released from the Li-Cu-HA/Col scaffold.

1. Introduction

More than 1.6 million people receive bone grafts because of tumor resection, pathological deformation, congenital deformation, sports injury, and infection treatment each year in the USA [1]. Critical-sized bone defect repair remains a real challenge in the field of orthopedics [1, 2]. Currently, stem cell-based tissue engineering reconstruction of bone defect is a feasible and continuously developing strategy to restore structure and function [3]. Bone marrow mesenchymal stem cells (BMSCs), a seed of bone regeneration, can differentiate into osteoblasts and secrete angiogenic factors to promote bone regeneration [4–6]. Thus, the BMSCs have potential to couple the osteogenesis and angiogenesis processes. Simultaneously, the trace elements were considered bone seekers due to their stable release kinetics, preferable stability in a biological environment, and low cost [7]. It had been demonstrated that many trace elements could promote osteogenesis or angiogenesis in bone regeneration [7]. Consequently, trace element release from the bioactive scaffold to couple the osteogenesis and angiogenesis of BMSCs are becoming a promising therapeutic strategy in bone tissue regeneration.

The osteogenesis of BMSCs involving many molecular signaling pathways and the canonical Wnt signaling pathway has been shown to be one of them. The Wnt signaling plays an important role in bone development, homeostasis, osteoblast differentiation, and bone formation [8]. Lithium can activate the canonical Wnt signaling pathway by inhibiting the activity of GSK3β, thereby inhibiting the phosphorylation and degradation of β-catenin [9]. Accumulated β-catenin is transported to the nucleus and initiates transcription of the osteogenesis gene (Wnt target genes) by binding to T cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription reporters [10]. In recent years, several studies indicated that the activation of the canonical Wnt signaling pathway could promote the migration and proliferation of bone marrow mesenchymal stem cells, adipose-derived stem cells and the osteogenic differentiation-related gene (Runt-related transcription factor 2, osterix, etc.) expression [11, 12]. Additionally, lithium-based scaffolds could stimulate bone marrow mesenchymal stem cells' osteogenesis and enhance bone regeneration via activation of the canonical Wnt signaling pathway [13, 14]. Therefore, lithium can be used as a promising bioactive trace element to promote the osteogenesis process.

The process of angiogenesis in a physiological and pathophysiological manner is highly related to the activation of the HIF-1α signaling pathway [15, 16]. Increasing evidence has been demonstrated that the expression of hypoxia-inducible factor 1α (HIF-1α) in mesenchymal stem cells enhances the VEGF expression and secretion to promote blood vessel formation [17]. Copper, an essential trace element in the human body, can inhibit the prolyl hydroxylation of HIF-1α by prolyl hydroxylases (PHDs) to stabilize HIF-1α expression [18]. Simultaneously, copper acts as a physiological inhibitor for FIH-1 (hydroxylase or factor inhibiting HIF-1α) [18]. The FIH had been demonstrated to inhibit the HIF-1α's interaction with the cofactors SRC-1, CBP, and p300 and then downgrade the expression of the HIF-1α target gene (VEGF, Glut1, etc.) [18]. In addition, copper-based scaffolds could upregulate the angiogenic factor levels and enhance the bone defect healing via activation of the HIF-1α signaling pathway [19]. Thus, copper is promising as an inducer of tissue engineering vascularized bone.

Interestingly, there are inextricable and extensive connections between canonical Wnt and HIF-1α signaling pathways. Kaidi and colleagues revealed that β-catenin could be used as a cotranscription factor to bind with HIF-1α and promote the expression of HIF-1α signaling pathway downstream genes [20]. While the role of HIF-1α in the Wnt signaling pathway is still controversial. In neurogenesis and bipolar disorder, Valvezan and Klein had reported that the HIF-1α promotes Wnt target gene expression in stem cells [21]. However, in colon cancer, hypoxia-activated HIF-1α inhibits β-catenin-T cell factor-4 (TCF-4) complex formation and transcriptional activity in hypoxia [20], while whether the canonical Wnt and HIF-1α signaling pathway activated by lithium and copper can couple and enhance the osteogenesis-angiogenesis in the BMSCs is still unclear.

In this study, we fabricated a lithium- (Li^+-) and copper- (Cu^{2+}-) doped organic/inorganic (Li-Cu-HA/Col) scaffold to explore their effects on coupling osteogenesis-angiogenesis. And then, we screened the optimal concentration of lithium and copper to evaluate their ability as an agonist of canonical Wnt and HIF-1α signaling pathways. Finally, we investigated the osteogenesis-angiogenesis coupling ability in BMSCs under the costimulation of optimal lithium and copper concentration and explore the underlying mechanism.

2. Materials and Methods

2.1. Isolation and Characterization of BMSCs. The BMSCs were isolated from 6-to-8-week old Balc/c mice. Femurs and tibias from mice were dissected, and the bone marrow was flushed with Dulbecco's Modified Eagle's Medium (DMEM; HyClone, USA). Bone marrow-derived cells were filtered through a 70 μm cell strainer (BD Falcon, USA) and collected by centrifugation at 250 g for 10 min. Resuspend the cells with 2 ml Ammonium-Chloride-Potassium Lysis Buffer (Beyotime, China), and add 6 ml DMEM to stop the reaction after 5 minutes. Then, centrifuge at 250 g for 5 minutes to obtain BMSCs, and the cells were cultured in DMEM, supplemented with 10% fetal bovine serum (Invitrogen-Gibco, USA) and 1% (v/v) antibiotic/antimycotic (Invitrogen-Gibco, USA). When cells grew to 80% confluence, 0.25% trypsin (Sigma-Aldrich, USA) was used to passage the cells.

BMSCs were seeded into 6-well plates and cultured in the complete medium. According to the manufacturer's instructions, osteogenic and adipogenic induction was stimulated by using commercial kits (Cyagen, China). Osteogenesis effects were detected by Alizarin Red staining (ARS; Sigma-Aldrich) after 3-week induction, and the Oil Red O was used for adipogenic detection after 3-week induction. Chondrogenic differentiation was induced using the micromass culture technique. BMSCs were collected and washed three times with chondrogenic induction medium (Cyagen, China) under centrifugation at 350 g for 5 min. The pellet was cultured in 0.5 ml chondrogenic induction medium in the

15 ml tube. After 2 days, the pellet was suspended by slightly knocking the bottom of the tube. The medium was replaced every two days, and the pellet was cultured for 28 days. Chondrogenesis effects were detected by Alcian blue staining.

2.2. Alizarin Red, Oil Red O, and Alcian Blue Staining. For Alizarin Red staining of cultured cells, BMSCs were washed with 1× Phosphate-Buffered Saline (PBS) and fixed with 10% formalin for 20 min. Then, cells were washed in tap water three times before staining with Alizarin Red solution (Sigma-Aldrich, USA) for 15 min. Finally, cells were rewashed with tap water three times and viewed under an inverted microscope (Nikon Eclipse TS100, USA).

For Oil Red O staining of cultured cells, BMSCs were washed with 1× PBS and fixed with 10% formalin for 20 min. Then, cells were washed in tap water three times before staining with Oil Red O solution (Sigma-Aldrich, USA) for 15 min. The excess stain was removed by washing with 70% ethanol three times. Finally, cells were rewashed with diluted water three times and viewed under an inverted microscope (Nikon Eclipse TS100, USA).

For Alcian blue staining, tissues generated from the pellet cultures were fixed in 10% formalin for 2 h and then washed twice with 1× PBS before the addition of 0.1% stock solutions of Alcian blue. After 30 min incubation at room temperature, the dye solution was removed and the constructs were washed with distilled water. Moreover, the staining results were recorded under an inverted microscope (Nikon Eclipse TS100, USA).

2.3. Fabrication of Lithium- and Copper-Doped Hydroxyapatite/Col Scaffold (Li-Cu-nHA/Col). We firstly synthesized lithium- and copper-doped hydroxyapatite (Li 2.5-Cu 1.0-HA) powder through a microwave-assisted hydrothermal method [22]. The Li 2.5-Cu 1.0-HA powder was prepared as follows: Ca $(NO_3)_2{\cdot}4H_2O$ and $NH_4H_2PO_4$ (Aldrich Chemical, USA) were dissolved in deionized water separately to form 0.1 M and 0.06 M solution. And then, add $LiNO_3$ and Cu $(NO_3)_2$ solid with designed Li/(Li+Cu +Ca) and Cu/(Li+Cu+Ca) molar ratios of 0.025 and 0.01, separately, to form the mix solution. Subsequently, the pH of the mix solution and $NH_4H_2PO_4$ solution was buffered close to 12 by $NH_3{\cdot}H_2O$, and then, pour the adjusted $NH_4H_2PO_4$ solution into Ca $(NO_3)_2{\cdot}4H_2O$ solution slowly. Then, the above resolution was transferred into an autoclave (60 ml) and heated in a microwave oven (MDS-6, Sineo, China) for 120 min at 130°C. After cooling, the products were collected by centrifugation, washed three times with deionized water and ethanol solution, and then dried at 60°C. The obtained products were labeled as Li 2.5-Cu 1.0-HA.

The porous ceramic structures of the Li 2.5-Cu 1.0-HA were produced and designed using the H_2O_2 foaming method [23]. Approximately 100 g of Li 2.5-Cu 1.0-HA powder, 7.5 ml of cellulose, 25 ml of H_2O_2, 7.5 ml of polyvinyl alcohol (PVA), and 60 ml of deionized water were mixed to form a ceramic slurry. This slurry was heated for 2 min in a microwave to generate gas and then molded to obtain the porous ceramic scaffold. Afterward, the scaffold was dried at 80°C for 12 h, following sintering at a heating rate of $5°C{\cdot}min^{-1}$ for 6 h until 1200°C, and then, the ceramics in the furnace were cooled until room temperature. All the samples were cut into $\Phi 10\,mm \times 10\,mm$ cylinders, and the Archimedes principle method was used to test the sintered Li 2.5-Cu 1.0-HA scaffolds' porosity.

And then, we used the vacuum infusion method, described by Zhou et al., to establish the Li 2.5-Cu 1.0-HA/Col scaffold according to the "brick-and-mortar" reinforcement theory [23]. We were dispersing the type I collagen (Sigma Chemical, USA) into 1.5 simulated body fluid (SBF) solution at a $10\,g{\cdot}ml^{-1}$ concentration to $20\,g{\cdot}ml^{-1}$, and it was adjusted using 5% $w{\cdot}v^{-1}$ acetic acid to a pH ranging from 4.0 to 6.5. After that, the scaffold was fully immersed into the type I collage solution and sealed in a high-pressure vessel. And then, the vacuum infusion with a pressure of 10 Pa was used to fill the collage I solution in the porous Li 2.5-Cu 1.0-HA scaffolds, which sustained for 2 h to allow the full saturation of the ceramic materials. Ultrasonic vibration and a repeated process were conducted for vacuum infusion. Finally, the Li 2.5-Cu 1.0-HA/Col scaffold was lyophilized at -20 C° and sterilized by γ-rays (^{60}Co) at a dose of 25 kGy for 1 h for use.

2.4. Characterization of the Li-Cu-HA/Col Scaffold. The chemical compositions of the scaffolds were analyzed by Fourier Transform Infrared Spectroscopy (FTIR; PerkinElmer Spectrum One B, USA) with scanning from $4000\,cm^{-1}$ to $500\,cm^{-1}$. The uniaxial compression testing was conducted to investigate the mechanical properties of HA, Li 2.5-Cu 1.0-HA, and Li 2.5-Cu 1.0-HA/Col scaffolds using a universal mechanical testing machine (Shimadzu-Series AGS-IX, Japan). Briefly, $\Phi 10\,mm \times 10\,mm$ scaffolds were fixed on the testing platen. And then, we used the load displacement measurements to obtain the stress-strain curve via a 10 kN load cell at a crosshead speed of $0.5\,mm{\cdot}min^{-1}$. After that, we got the maximum compressive strength (maximum load) through the stress-strain curve. In addition, the scaffolds' microstructural characterization was observed using scanning electron microscopy (Electron Co., Japan).

The porosity of Li 2.5-Cu 1.0-HA and Li 2.5-Cu 1.0-HA/Col scaffolds was determined by using the Archimedes principle method. We measure three kinds of weights ($w1$, $w2$, and $w3$) under different conditions. $w1$ is the mass of the dried sample (30 min vacuum treatment), $w2$ is the mass of the saturated sample weighed in the air, and $w3$ is the apparent mass of the saturated sample weighed in liquid. And then, we use Equation (1) to calculate the porosity.

$$\text{Porosity} = \frac{w2 - w1}{w3 - w2} 100\%. \quad (1)$$

The solution-mediated degradation properties of scaffolds were tested in the simulated body fluid (SBF). HA/Col and Li 2.5-Cu 1.0-HA/Col scaffolds ($n = 3$, per concentration) were placed in clean vial bottles and completely immersed in 5 ml of SBF. Then, the bottles were sealed and put into a 37°C water bathing constant temperature vibrator, and the SBF was refreshed every 3 days. The

scaffolds were taken out at designated time intervals of 2, 4, 7, 10, 14, 17, 21, 28, and 30 days. An obsolete release medium was gathered for measuring ionized lithium/copper/phosphate concentrations.

2.5. Live-Dead Cell Staining and MTS Assay of BMSCs Cocultured with the Scaffold. Before BMSC seeding, all the scaffolds were soaked in culture medium for 24 h at 37°C in a humidified incubator with 5% CO_2. P3 BMSCs were seeded dropwise on the scaffolds after removing the culture medium. Subsequently, the cell-seeded scaffolds were kept at 37°C in a humidified incubator with 5% CO_2 for 2 h to allow cells to attach to the scaffolds. Finally, the cell-seeded scaffolds were cultured in the culture medium.

The cell proliferation rate of BMSCs on the scaffold was measured using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, USA) on day 1, 3, 5, and 7 time points. The BMSC coculture scaffolds were washed twice with PBS and incubated with 200 μl MTS solution plus 800 μl culture medium for 3 h in a humidified incubator. After that, the absorbance of the culture medium was read by using Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek Instruments, Inc., USA) at 490 nm.

After coculture of the scaffold with P3 BMSCs for 3 days, a Live-Dead Cell Staining Kit (calcein-AM/PI double staining kit, Taiwan, China) was used for detecting the dead cells. The BMSC coculture scaffolds were washed twice with PBS and stained with Live-Dye (a green fluorescent dye for live cells, Ex/Em = 488/518 nm) and propidium iodide (PI, a red fluorescent dye, Ex/Em = 488/615 nm). After incubation for 15 min at 37°C, live and dead cells were visualized by fluorescence microscopy (Leica TCS SPS, Leica, Germany).

2.6. ELISA for Cytokine Detection. Take the sample culture medium into a centrifuge tube and centrifuge at 3000 rpm for 10 minutes at 4°C. Collect the upper layer of medium and freeze it at -80° for use. The alkaline phosphatase (ALP), osteocalcin (OCN), and VEGF protein concentration in the culture medium were detected using an enzyme-linked immunosorbent assay kit (Baolai Biotech, China). The test was performed on five samples per treatment, according to the manufacturer's protocol.

2.7. Cell Proliferation by MTS Assay and Cell Count. BMSCs at P3 were cultured under different interventions for 1, 3, 5, and 7 days. And then, CellTiter 96R AQ_{ueous} One Solution (MTS, Promega Corporation, USA) was added to each well in a 1 : 10 dilution in the culture medium. After incubation for 4 h at 37°C and 5% CO_2 in the humidified atmosphere, absorbance was measured in a microplate reader at 490 nm (Molecular Devices, USA). Simultaneously, the BMSC number was counted by using Cellometer Mini Automated Cell Counter (Nexcelom Bioscience, USA) on day 7.

2.8. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) and Western Blot (WB) Analysis. Total RNA was subsequently extracted using the RNeasy Mini Kit following the manufacturer's instructions (Qiagen, USA) and reverse-transcribed into cDNAs using SuperScript IV Reverse Transcriptase (Thermo Fisher, USA). RT-qPCR was performed in an ABI StepOnePlus™ instrument (Applied Biosystems, USA) using the SYBR® Premix Ex Taq™ (Takara, Japan). Each RT-qPCR analysis was performed using six independent biological samples. Relative mRNA expression levels were calculated using the $2^{\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a housekeeping control. The primers used for the RT-qPCR are listed in Supplementary Table 1.

For western blotting, BMSCs were harvested with radioimmunoprecipitation assay lysis buffer (Sigma, USA) supplemented with protease inhibitor cocktail (1 : 100, Sigma, USA). Protein concentrations were determined using a protein assay kit (Thermo Fisher, USA) according to the manufacturer's instruction. The Invitrogen NuPage® Novex® Gel System was used for protein separation in 4–12% Bis-Tris Protein Gels at 200 V for 50 min. Proteins were then transferred to a Polyvinylidene Fluoride (PVDF) membrane by using iBlot™ Transfer Stack. Membranes were blocked in 5% nonfat milk in the TBST (a mixture of tris-buffered saline (TBS, Thermo Fisher, USA) and 0.1% polysorbate 20 (Tween, Thermo Fisher, USA)) for 1 h, followed by incubation with a primary antibody (diluted in 1% nonfat milk in 0.1% TBST) overnight at 4°C. After washing, membranes were incubated with HRP-conjugated secondary antibodies (Abcam, USA) for 1.5 h at room temperature, washed with 0.1% TBST, and imaged with the ChemiDoc™ Touch Imaging System (Bio-Rad, USA). Antibody information is provided in Supplementary Table 2.

2.9. Chromatin Immunoprecipitation (ChIP). The chromatin immunoprecipitation (ChIP) assay was performed as described previously [24]. Briefly, the cells were incubated in 1% formaldehyde for 10 min at 37°C for protein crosslinking to DNA. Following this, cells were collected and lysed in sodium dodecyl sulfate lysis buffer, and 200-1000 bp DNA fragments were obtained by using a Branson Sonifier 450 (PA, USA) under the following condition: six times for periods of 15 s each. After centrifuging, the supernatant containing chromatin was diluted 100-fold, and an aliquot (2% volume) was used to indicate the input DNA amount in each sample. The remaining chromatin extract was incubated with anti-β-catenin, anti-HIF-1α, and anti-IgG (negative control) using salmon sperm DNA/protein A agarose beads overnight at 4°C with gentle rotation. Crosslinking was reversed for 4 h at 65°C and was followed by proteinase K digestion. DNA was purified by a standard phenol/chloroform method, reverse transcriptased, and subjected to real-time PCR. Primer sequences are available in Supplementary Table 2.

2.10. Immunofluorescence (IF) Staining. For IF analysis of cultured cells, BMSCs were fixed with 10% formalin for 15 minutes. Permeabilize cells with 0.1%. And then, they were blocked in PBS with 5% bovine serum albumin for 30 minutes at room temperature. Immunostaining was performed using the primary antibody against β-catenin and VEGF and the secondary antibody against rabbit or mouse IgG. Nuclei were counterstained with 4′,6-diamidino-2-

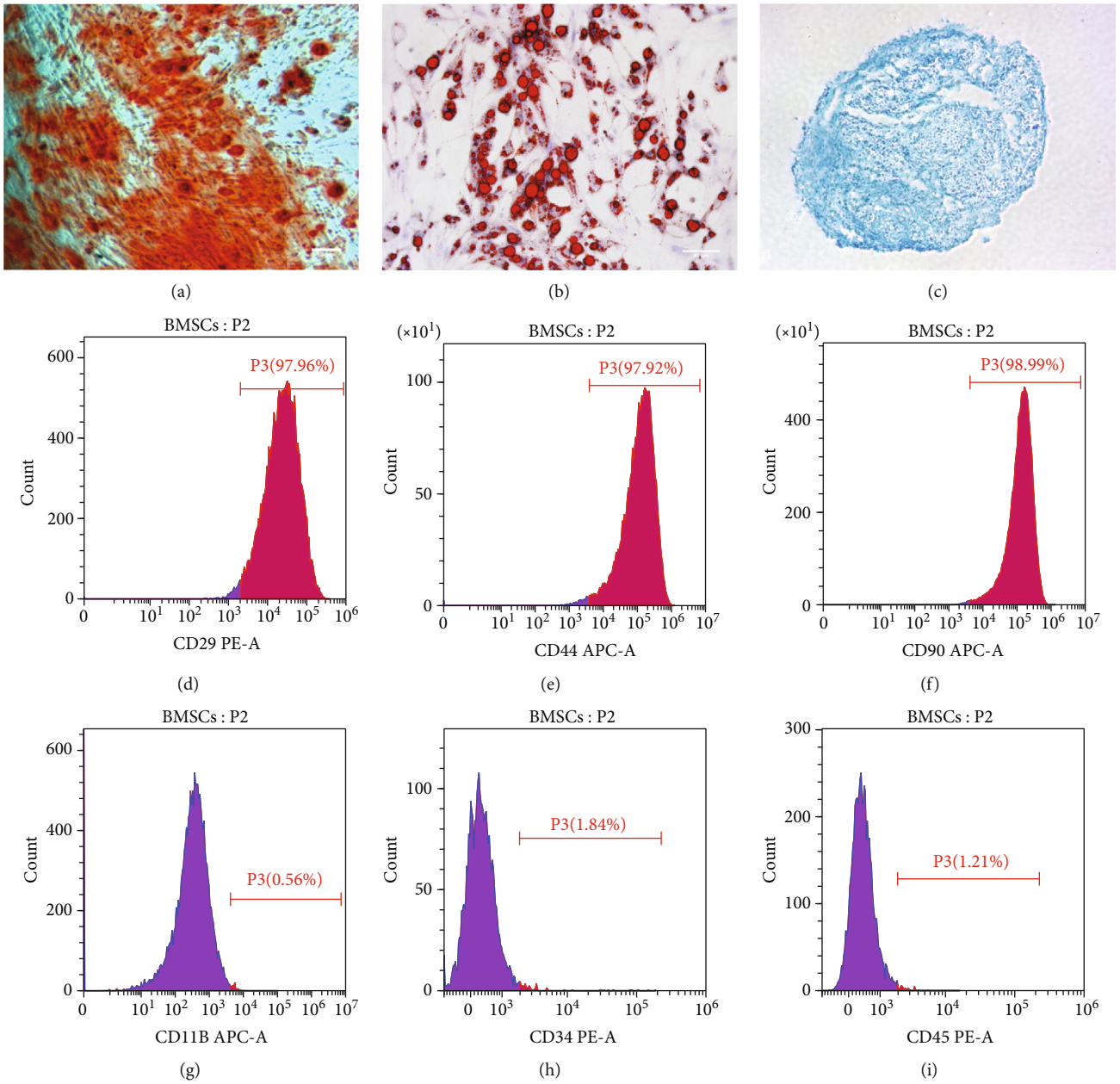

Figure 1: Multiline differentiation and surface markers of BMSCs. (a) Osteogenic differentiation. The Alizarin Red staining result showed that the BMSCs formed a massive red calcium nodule after three weeks of osteogenesis induction. (b) Adipogenesis differentiation. After three weeks of adipogenesis induction, many red lipid droplets can be seen in the cells. (c) Chondrogenesis differentiation. The Alcian blue staining of chondrogenic pellets confirmed the accumulation of extracellular matrix proteins in the chondrogenic pellet. (d–i) The cells positively express CD29, CD44, and CD90, while they have negative expression of CD11b, CD34, and CD45.

phenylindole (DAPI). Images were captured using a fluorescence microscope (Leica TCS SPS, Leica, Germany).

2.11. Statistical Analysis. The GraphPad Prism 7.0 software (San Diego, CA) was used for the statistical analyses. Data are presented as the mean ± standard error (SE). One-way ANOVA was used for the comparison between multigroups' results, followed by the comparison between each two groups using the Tukey analysis. ∗ indicates a P value < 0.05, ∗∗ indicates a P value < 0.01, and ∗∗∗ indicates a P value < 0.001.

3. Results

3.1. Identification of BMSCs. To characterize cells isolated by the adherence method, we detected the osteogenesis, adipogenesis, and chondrogenesis potential and surface markers of mouse bone marrow mesenchymal stem cells (BMSCs). The Alizarin Red, Oil Red O, and Alcian blue staining results revealed that the BMSCs had osteogenesis, adipogenesis, and chondrogenesis differentiation potential (Figures 1(a)–1(c)). The cells positively express CD29, CD44, and CD90, while

negative for CD11b, CD34, and CD45, which is consistent with the characteristics of stem cells (Figures 1(d)–1(j)).

3.2. Li-Cu-HA/Col Scaffold Has an Excellent Physicochemical Property. We successfully used the microwave-assisted hydrothermal method to fabricate the Li-Cu-HA/Col scaffold. The FTIR results showed that the ceramic powder (HA, Li 2.5-HA, Cu 1.0-HA, and Li 2.5-Cu 1.0-HA) had absorption peaks of hydroxyapatite, and the Li 2.5-Cu 1.0-HA/Col powders also presented the absorption peaks of collagen (Figure 2(a)). After that, we established the Li 2.5-Cu 1.0-HA and Li 2.5-Cu 1.0-HA/Col scaffolds by the foaming and vacuum infusion method and found that the porosity of Li 2.5-Cu 1.0-HA ($75.5 \pm 8.5\%$) was significantly higher than that of the Li 2.5-Cu 1.0-HA/Col ($52.4 \pm 6.8\%$) scaffold, which reflected the higher compaction of Li 2.5-Cu 1.0-HA/Col. Simultaneously, Li 2.5-Cu 1.0-HA/Col was also superior in mechanical strength to the Li 2.5-Cu 1.0-HA and HA scaffolds (Figure 2(b)). At the microstructure level, the Li 2.5-Cu 1.0-HA ceramic's pore size was 200 μm to 400 μm (Figures 2(c) (1)), and the vacuum infusion process forces Collagen I to fill the ceramics' porous matrix (Figure 2(c) (2), (3), and (4)). In the scaffold degradation experiment, the Li^+, Cu^{2+}, and phosphate ions were slowly released from the Li 2.5-Cu 1.0-HA/Col scaffold. Due to the adsorption of collagen on the surface of the ceramic scaffold, the release rate of trace element and phosphate ions was relatively slow in the early phase. And then, the release rate gradually increased and reached a stable rate after 7 days (Figure 2(d)), while, compared to the HA/Col scaffold, the phosphate ions released from the Li 2.5-Cu 1.0-HA/Col scaffolds were significantly reduced.

3.3. Li-Cu-HA/Col Scaffold Can Promote the Process of Osteogenesis and Angiogenesis. Based on the excellent physical and chemical properties of Li 2.5-Cu 1.0-HA/Col, we further explored the biocompatibility and osteogenic and angiogenic properties of the scaffold. As expected, the Li 2.5-Cu 1.0-HA/Col scaffold promotes the proliferation of BMSCs and benefits the vitality of BMSCs (Figures 3(a) and 3(b)). Moreover, the ability of BMSCs to secrete osteogenesis- (ALP, OCN) and angiogenesis- (VEGF) related factors were significantly enhanced when cocultured with the Li 2.5-Cu 1.0-HA/Col scaffold in vitro (Figure 3(c)).

3.4. Lithium (500 μM) Can Rescue the Inhibited Wnt Signaling Pathway. To gain insights into the role of lithium in the canonical Wnt signaling pathway, we examined the proliferation of BMSCs under the stimulation of lithium and further explored whether the optimal lithium concentration can effectively rescue the inhibited Wnt signaling pathway. Low concentration of Li^+ (125 μM-1 mM) was proven to be beneficial to BMSCs' proliferation, and 500 μM was the optimal stimulation concentration, which was confirmed by the MTS assay (Figure 4(a)) and cell counts (Figure 4(b)). Importantly, 500 μM Li^+ can activate the canonical Wnt signaling pathway in BMSCs. RT-qPCR and western blot analysis revealed that the β-catenin and osterix expression was increased and p-GSK3β was drastically declined in the BMSCs under the stimulation of 500 μM lithium (Figures 4(c) and 4(d), bar 1 versus bar 2). Notably, lithium (500 μM) can rescue the inhibitory effect of 10 μM XAV-939 on the canonical Wnt signaling pathway in BMSCs. On the 500 μM lithium and 10 μM XAV-939 costimulation group, the expression of β-catenin, osterix, and p-GSK3β was similar to that of the Wnt signaling pathway-activated group (Figures 4(c) and 4(d), bar 2 versus bar 3). Compared with the 10 μM XAV-939 stimulation group, the expression of β-catenin and osterix was drastically elevated in the combination group in BMSCs. At the same time, the p-GSK3β was significantly decreased (Figures 4(c) and 4(d), bar 3 versus bar 4). Moreover, we got similar results in immunofluorescence staining of β-catenin in BMSCs under the different stimulations (Figure 4(e)).

3.5. 25 μM Copper Is an Appropriate Concentration to Activate the HIF-1α Signaling Pathway. To determine copper's role in the HIF-1α signal pathway, we examined VEGF expression under the stimulation of copper in BMSCs. Further, we explored the efficacy of copper on the activation of the HIF-1α signal pathway. A low copper ion concentration (5 μM-100 μM) can promote the VEGF expression, and 25 μM copper ions have the same efficacy with 1% oxygen microenvironment on stimulating the secretion of VEGF (Figure 5(a)). In addition, under copper stimulation, the expression of HIF-1α and VEGF was significantly revealed than that of the control group (Figures 5(b) and 5(c), bar 1 versus bar 2), and their expression levels were equivalent to 1% hypoxic environment (Figures 5(b) and 5(c), bar 2 versus bar 3). Moreover, we got similar results in immunofluorescence staining of VEGF in BMSCs under the different stimulations (Figure 5(d)).

3.6. Lithium and Copper Costimulation Can Promote Osteogenesis and Angiogenesis via Crosstalk between the Canonical Wnt and HIF-1α Signaling Pathways. A previous study had revealed that the canonical Wnt and HIF-1α signaling pathways had inextricable and extensive connections [20, 21], although we found that 500 μM Li^+ and 25 μM Cu^{2+} could effectively activate the canonical Wnt and HIF-1α signaling pathway in BMSCs, respectively. However, whether the lithium and copper could couple and enhance the osteogenesis and angiogenesis and the underlying mechanism are still unknown. So, we used the 500 μM Li^+ and 25 μM Cu^{2+} to costimulate the BMSCs and found that the number of calcium nodules and VEGF-positive expression cells are significantly increased compared with those in 500 μM Li^+ or 25 μM Cu^{2+} stimulation (Figure 6(a)). Simultaneously, the RT-qPCR and western blot had demonstrated that the osteogenesis- (ALP, osterix) and angiogenesis- (VEGF) related factors were significantly increased in the coculture group (Figures 6(b) and 6(c)). All these suggested that the osteogenesis and angiogenesis ability of BMSCs in the costimulation group was enhanced. Furthermore, Kaidi et al. reported that β-catenin could combine with HIF-1α to promote the VEGF and Glut 1 expression [20]. And Wan et al. have reported that HIF-1α could bind to the promoter region of osterix and promote osteoblast differentiation in MSCs [25]. So, we used the ChIP analysis which demonstrated that the osterix gene promoter

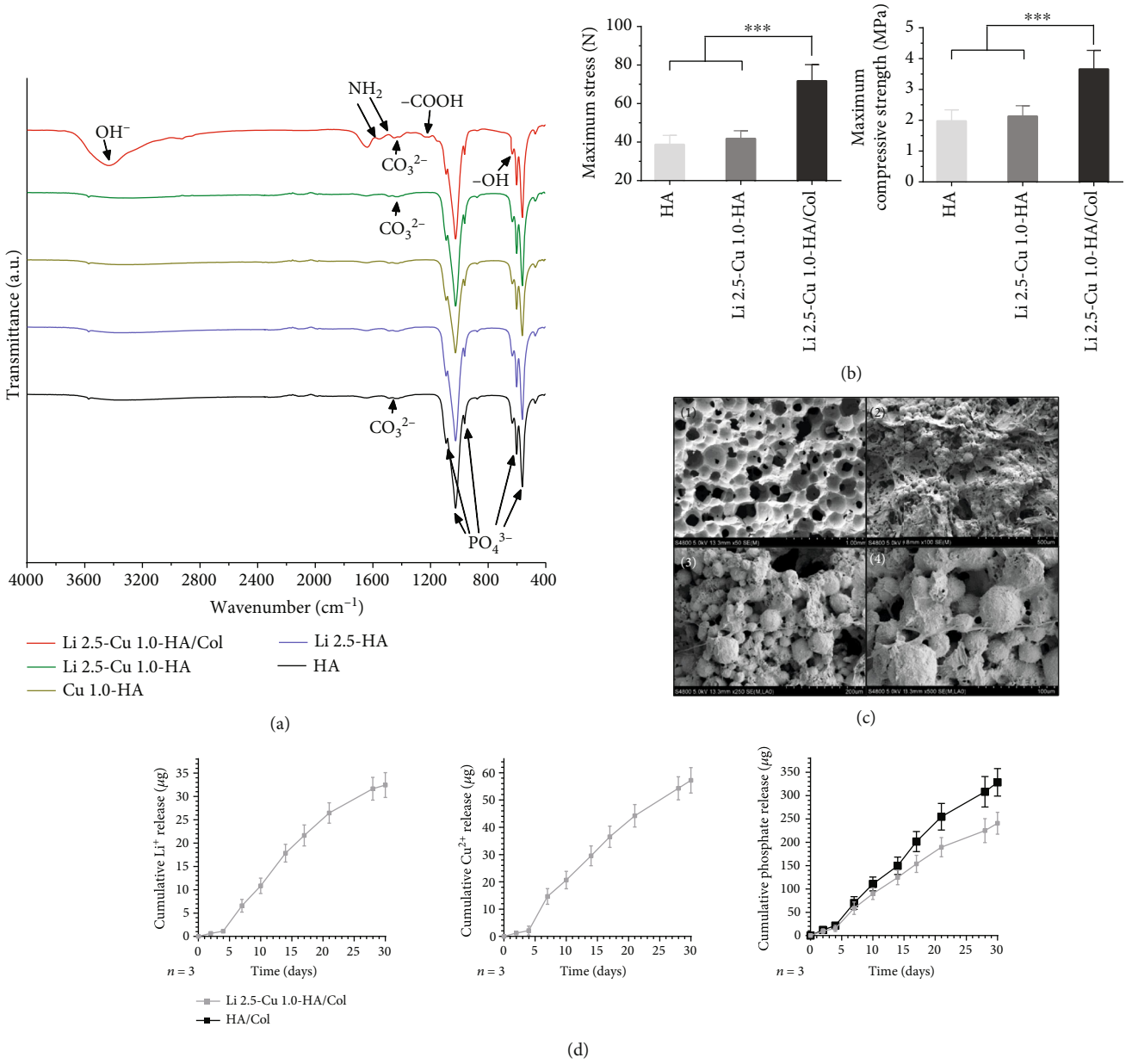

Figure 2: Li 2.5-Cu 1.0-HA/Col scaffold has excellent physicochemical properties. (a) FTIR analysis results showed that the ceramic powder (HA, Li 2.5-HA, Cu 1.0-HA, and Li 2.5-Cu 1.0-HA) had the PO_4^{3-} (1089, 1044, 962, 601, and 570 cm^{-1}) and OH^- (3571 and 633 cm^{-1}) absorption peak for hydroxyapatite and the NH_2 (1680 cm^{-1} to 1630 cm^{-1} and 1570 cm^{-1} to 1510 cm^{-1}) and -COOH (1339 cm^{-1}) absorption peak for Li 2.5-Cu 1.0-HA/Col. (b) The comparison of maximum stress and maximum compression strength between Li 2.5-Cu 1.0-HA and Li 2.5-Cu 1.0-HA/Col. (c) SEM scan of Li 2.5-Cu 1.0-HA and Li 2.5-Cu 1.0-HA/Col scaffolds. The Li 2.5-Cu 1.0-HA scaffold had a porous structure with a pore size of 200-400 μm (c, (1)). After vacuum infusion of type I collagen, the ceramic scaffolds' surface was covered with collagen I fibers and microspheres (c, (2)–(4)). (d) The Li^+, Cu^{2+}, and phosphate release curve of the Li 2.5-Cu 1.0-HA/Col scaffold under simulated body fluid (SBF) microenvironment.

region has obvious HIF-1α enrichment under the costimulation group and 25 μM Cu^{2+} in the BMSCs (Figure 6(d), bar 3 versus bar 2, bar 1). Simultaneously, there was β-catenin enriched in VEGF's promoter region in the costimulation group and 500 μM Li^+ in the BMSCs (Figure 6(e)). Accordingly, the costimulation of lithium and copper could crosstalk between the canonical Wnt and HIF-1α signaling pathways in the BMSCs and enhance the coupling of osteogenesis and angiogenesis.

4. Discussion

More and more evidence showed that trace elements (lithium, magnesium, zinc, copper, etc.) released from the bioactive scaffold can promote osteogenesis or angiogenesis in bone regeneration [7, 13, 26]. Increasing evidence revealed that the dual-delivery scaffold could couple osteogenesis and angiogenesis to promote bone regeneration [27, 28]. We have recently discovered that lithium and copper

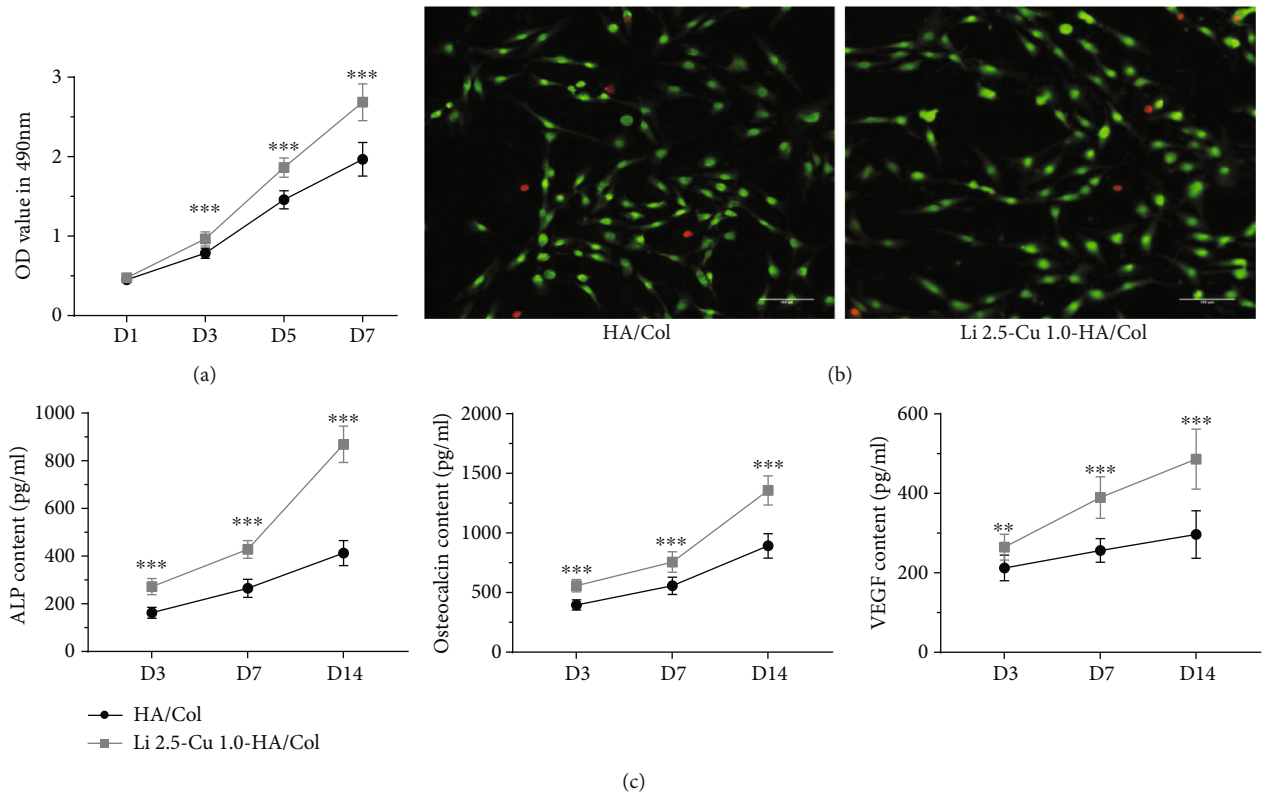

Figure 3: Li 2.5-Cu 1.0-HA/Col scaffold has good biocompatibility and could promote the secretion of osteogenesis and angiogenesis factors. (a, b) The proliferation and vitality of BMSC coculture with Li 2.5-Cu 1.0-HA/Col scaffolds tested by the MTS assay (days 1, 3, 5, and 7) and Live-Dead Cell staining (day 3). (c) ELISA analysis of the secretion of osteogenesis (ALP, OCN) and angiogenesis (VEGF) factors when BMSCs were cocultured with Li 2.5-Cu 1.0-HA/Col scaffolds.

sustainably released from the hydroxyapatite/collagen scaffold (Li 2.5-Cu 1.0-HA/Col) could promote the expression of osteogenesis- and angiogenesis-related factors when cocultured with BMSCs. This revealed that lithium and copper could couple the osteogenesis and angiogenesis of BMSCs. Therefore, clarifying the mechanism of lithium and copper in coupling osteogenesis and angiogenesis in BMSCs will provide a new strategy for bone defect repair. In this study, we further identified that the 500 μM lithium and 25 μM copper could effectively activate the canonical Wnt and HIF-1α signaling pathway. In addition, we demonstrated that crosstalk between the canonical Wnt and HIF-1α signaling pathways could couple and enhance the osteogenesis and angiogenesis in BMSCs under the costimulation of lithium and copper.

The normal bone repair involves multiple overlapping processes such as inflammation, osteogenesis, and vascularization et al. The coupling of osteogenesis and angiogenesis is an indispensable part of bone regeneration and repair. More and more studies have shown that the dual-delivery scaffold coupling osteogenesis and angiogenesis is a feasible method for bone regeneration [27, 28]. In our study, we fabricated the Li-Cu-HA/Col scaffold using a microwave-assisted hydrothermal combined with, foaming, and vacuum infusion method [22, 23]. With the deposition of type I collagen on the Li-Cu-HA scaffold's surface, the scaffolds' compressive stretch has reached the trabecular bone requirement (3.65 MPa), and the porosity that had been demonstrated can promote bone ingrowth [29]. Simultaneously, the Li-Cu-HA/Col scaffold had good biocompatibility and could encourage the proliferation of BMSCs. More importantly, the lithium and copper could sustain the release from the scaffold and promote osteogenesis and angiogenesis when cocultured with BMSCs. Previously, some scholars have doped two or more trace elements into the scaffold and proved that it could couple osteogenesis and angiogenesis and enhance bone repair. Bose and colleagues designed a magnesium- and silicon-doped 3D printed tricalcium phosphate scaffold in which magnesium and silicon were used to promote angiogenesis and osteogenesis, respectively. And they investigated that the scaffold could promote the expression of ALP, OCN, and VEGF to enhance the bone defect healing [27]. Weng et al. had been doping strontium and copper into the scaffold to play the role of osteogenesis and angiogenesis. And they demonstrated that the scaffold could couple the process of osteogenesis and angiogenesis to promote bone formation [28]. Thus, we believe that a lithium- and copper-doped scaffold with osteogenesis-angiogenesis coupling is promising as a new method for the treatment of bone defects.

Lithium has been used as a medicine to treat psychiatric patients for more than half a century, and it can activate the canonical Wnt signaling pathway to promote the MSC proliferation, migration, osteogenesis, etc. [30–32]. According to previous studies, low-concentration lithium was often

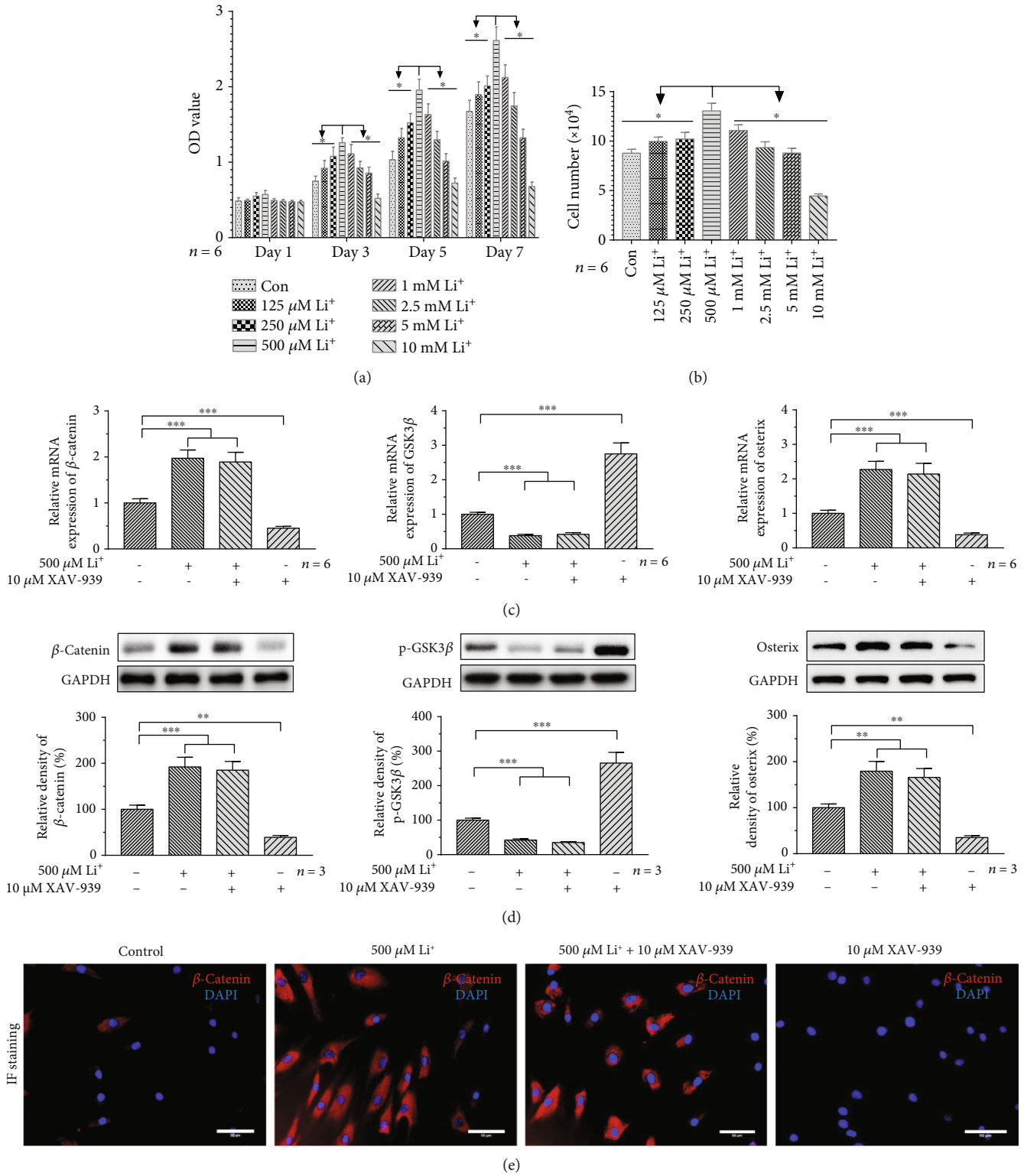

Figure 4: Lithium (500 μM) can rescue the inhibited canonical Wnt signaling pathway. (a) MTT assay of BMSC proliferation under the stimulation of different lithium ion concentrations. The OD values of BMSCs were recorded on days 1, 3, 5, and 7 after stimulation. $*$ means $P < 0.05$. (b) Cell count of BMSCs under the stimulation of different lithium ion concentrations for 7 days. Data are representative of six independent experiments, and $*$ means $P < 0.05$. (c, d) The expression of the Wnt signaling pathway-related gene (β-catenin, osterix, and p-GSK-3β) was measured by RT-qPCR and western blot in the BMSCs under the 500 μM Li^+, 10 μM XAV-939, and 500 μM Li^+ combined with 10 μM XAV-939 stimulation for 7 days. The results are depicted as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (e) The immunofluorescence staining of β-catenin in BMSCs under different stimulations for 7 days.

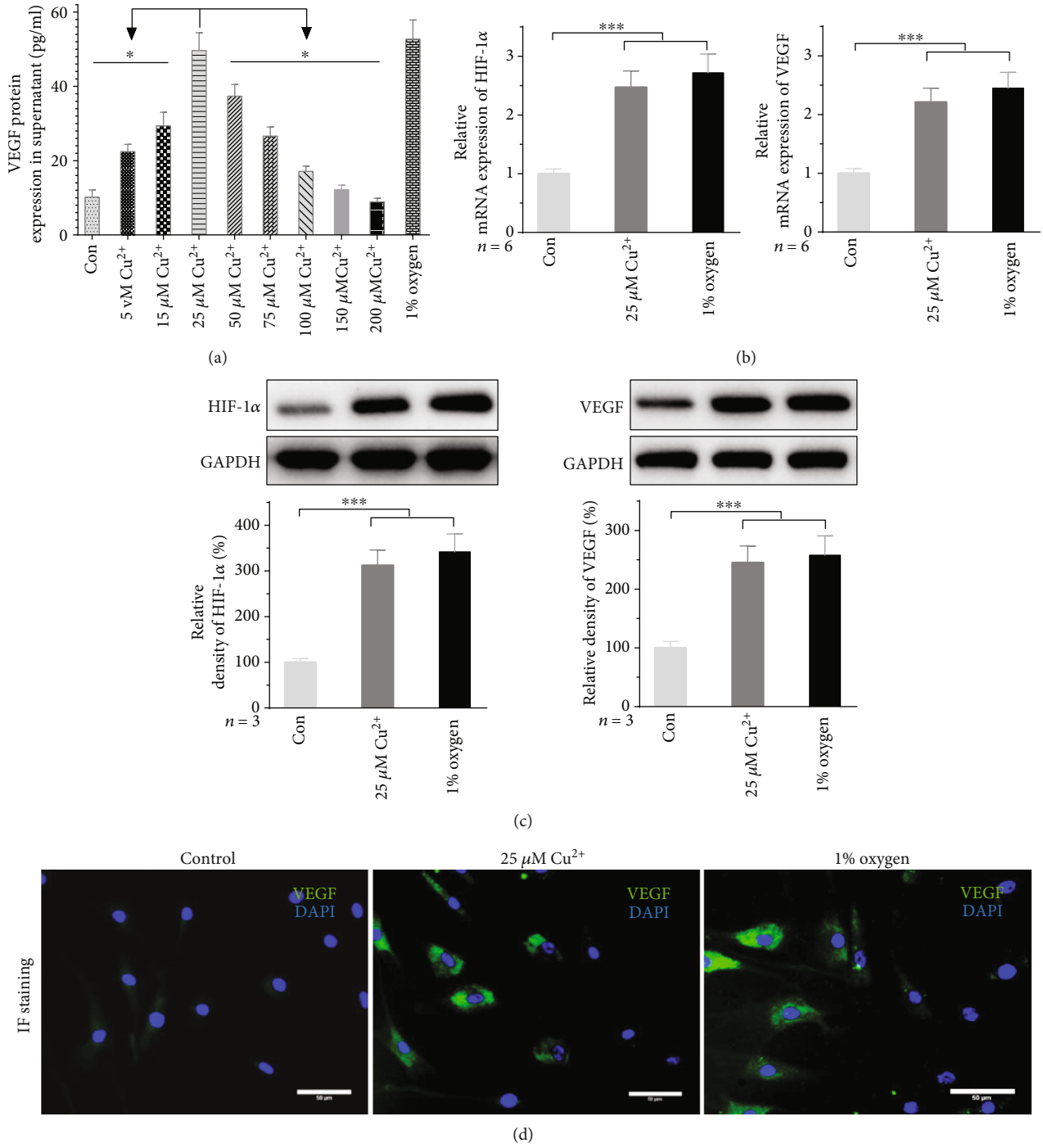

Figure 5: 25 μM copper is an appropriate concentration to activate the HIF-1α signaling pathway. (a) ELISA detected VEGF secretion on the different copper ions and 1% hypoxia niche stimulation. $*$ means $P < 0.05$. (b, c) The expression of the HIF-1α signaling pathway-related gene (HIF-1α, VEGF) was measured by RT-qPCR and western blot in the BMSCs under the 25 μM Cu^{2+} stimulation and 1% hypoxia intervention for 7 days. The results are depicted as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < .001$. (d) The IF staining of VEGF in BMSCs under the different stimulations for 7 days.

used to stimulate mesenchymal stem cell proliferation and osteogenic differentiation [31–33], while accumulation or high-concentration of lithium could inhibit the osteogenesis [34–36]. Tang et al. had investigated that lithium chloride (200 mg·kg^{-1}) gavage-fed to the rat for 2 weeks could promote the bone formation in the area of periapical bone lesions, while continuous lithium chloride treatment for 4 weeks impaired periapical bone healing [36]. Consistent with these findings, we investigated that BMSCs underwent proliferation and osteogenesis differentiation in low-concentration stimulation of lithium. In addition, we further revealed that 500 μM lithium rescued the inhibition effect of 10 μM

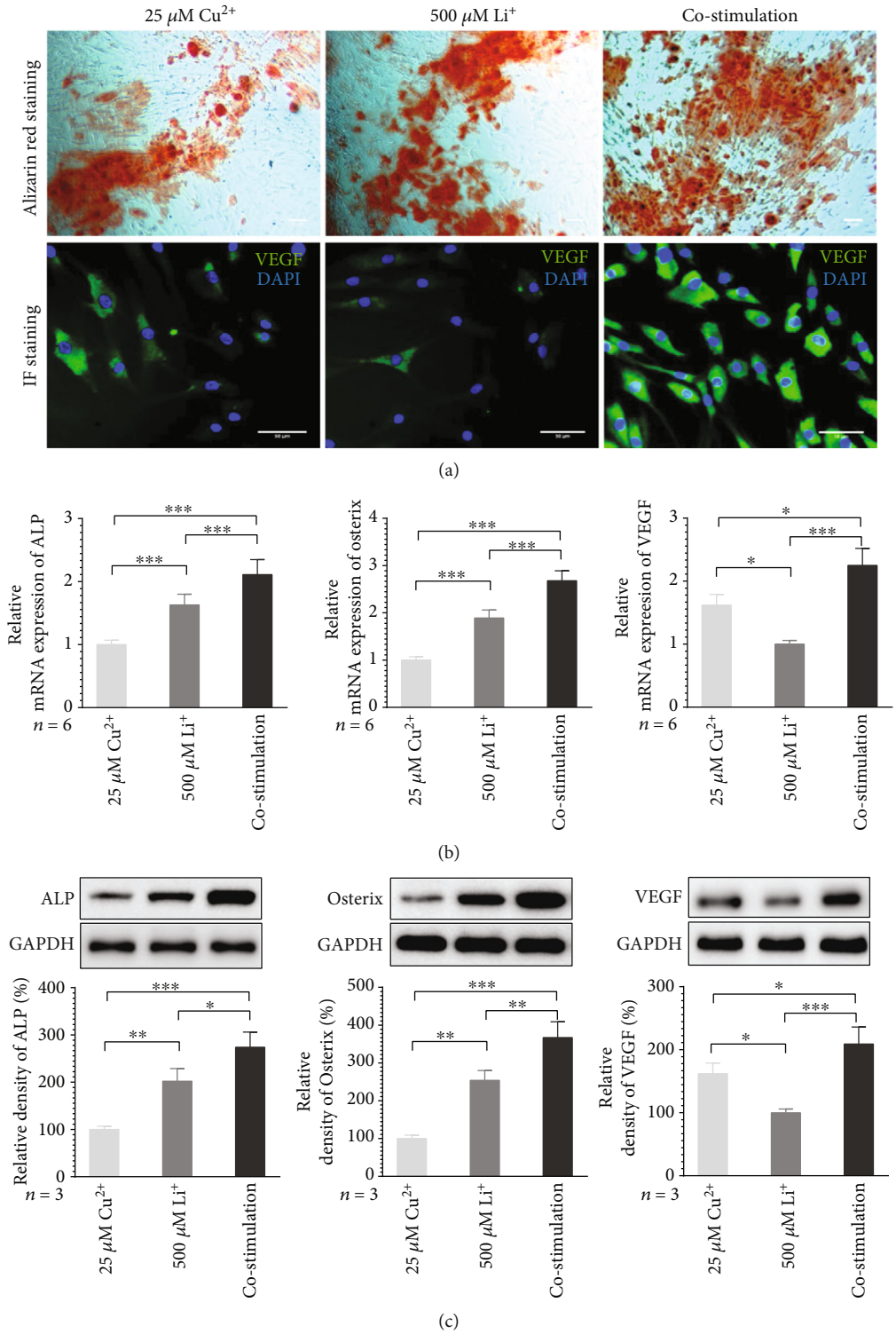

Figure 6: Continued.

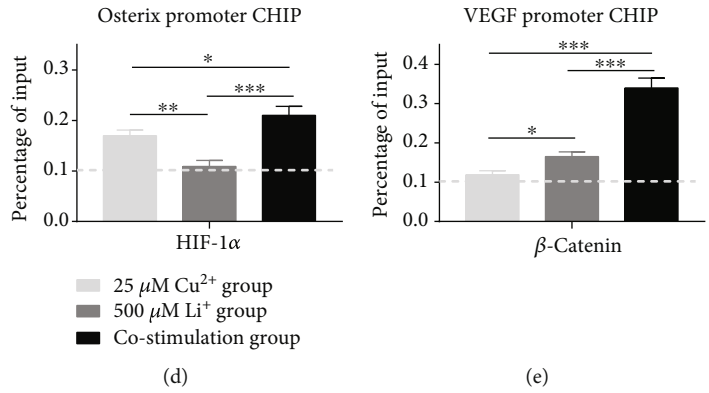

Figure 6: Lithium and copper costimulation can promote osteogenesis and angiogenesis via crosstalking between the canonical Wnt and HIF-1α signaling pathways. (a) The BMSCs were exposed to 25 μM copper, 500 μM lithium, and costimulation. The Alizarin Red staining was performed to investigate the calcium nodules after four weeks of stimulation. The immunofluorescence (IF) staining was used to detect the VEGF expression in the BMSCs after 7 days of coculture. (b, c) The osteogenesis (ALP, osterix) and angiogenesis (VEGF) genes were detected by RT-qPCR and western blot in the BMSCs under 25 μM Cu^{2+}, 500 μM Li^{+}, and costimulation intervention for 7 days. (d, e) ChIP analysis for HIF-1α, β-catenin, osterix, and VEGF promoter binding site following stimulation with 25 μM copper and 500 μM lithium or costimulation for 7 days. $n = 5$ holes per group. Data shown are of a single representative experiment with an experiment repeated two times with three technical replicates in each experiment. The results are depicted as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

XAV-939 on the canonical Wnt signaling pathway and osteogenesis differentiation in BMSCs. XAV-939 has been widely proven to be an inhibitor of the Wnt signaling pathway [37]. Recently, emerging evidence had revealed that XAV-939 could suppress the osteogenesis process via inhibiting the canonical Wnt signaling pathway [37, 38]. Yang and colleagues had reported that XAV-939 could inhibit the osteoprotegerin expression in preosteoblast cells and wnt3a (canonical Wnt agonist) could restore the effect [39]. Therefore, lithium could effectively activate the Wnt signal pathway at a concentration of 500 μM.

Copper is well known to be an essential trace element needed by the human body. A significant amount of information has been gained from understanding its role in angiogenic processes associated with tumor development [40]. In BMSCs, it had been demonstrated that copper can upregulate the HIF-1α signaling pathway to enhance the VEGF secretion and blood vessel formation [41, 42]. In our study, we investigated that the low-concentration copper could enhance the secretion of VEGF in the BMSCs. Moreover, we found that the 25 μM copper has the similar effect with 1% oxygen condition on the activation of the HIF-1α signaling pathway. One percentage of oxygen microenvironment is a lower oxygen tension than the physoxic state of most tissue and organs [43, 44]. And the HIF-1α would sustain expression and promote the VEGF expression in cells under the 1% oxygen stimulation [45, 46]. Simultaneously, Highet et al. reported that HIF-1α was mainly in the cytoplasm under 5% hypoxia condition and transferred to the nucleus when stimulated under 1% hypoxia condition [47]. The ectopic nuclear HIF-1 can upregulate its target genes. Thus, we believed that 25 μM copper is an appropriate concentration to activate the HIF-1α signaling pathway in BMSCs.

It is common knowledge that there are extensive connections and communications between intracellular signals. Previous studies had shown that the expression of proteins such as c-Myc and AKT downstream of the Wnt signaling pathway could upregulate the expression of HIF-1α and promote angiogenesis [48], while the activation of the HIF-1α signaling pathway can promote the differentiation of stem cells to the direction of osteogenesis and promote the expression of osteogenesis-related genes by combining with transcription promoters such as HRE in the gene promoter region [49, 50]. Moreover, increasing evidence revealed that β-catenin has a direct-acting effect on the HIF-1α target gene. Kaidi et al. reported that there is an overlap between β-catenin- and HIF-1α-regulated genes. As cotranscription factors, β-catenin and HIF-1α bind to the promoter region of angiogenesis-related factors to promote the expression of VEGF, Glut 1, etc. [20]. In addition, Clifford et al. used the gene reporter constructs which identified that VEGF contains two T cell factor- (TCF-) binding sites, and β-catenin could bind to the TCF to upregulate the VEGF expression [51]. Consistent with these findings, our result demonstrated that, under the costimulation of lithium (500uM) and copper (25uM), the β-catenin could combine in the promoter region of VEGF to enhance its expression in BMSCs. In addition, the osteogenesis of BMSCs was also enhanced under costimulation. And we investigated that the osterix promoter was enriched with HIF-1α and the expression of osterix significantly upregulated under the costimulation. Osterix is a zinc finger-containing transcription factor encoded by the Sp7 gene, which can regulate the osteoblast differentiation of BMSCs [52]. As a master regulator of osteogenesis differentiation of BMSCs, osterix plays a key role in the regulation of collagen I, alkaline phosphatase (ALP), and osteocalcin (OCN) gene expression [52]. The osterix-silenced mice present complete absence of bone formation and completely arrested osteoblast differentiation accompanying lack of early and late markers of osteoblast differentiation, resulting in the perinatal lethality [53]. This is indirectly suggesting that osterix has a positive effect on promoting differentiation. Moreover, Wan et al. revealed that

the osterix has two consensus hypoxia-responsive elements (HRE-binding motifs) in the proximal promoter using in situ hybridization technology, and the HIF-1α could activate the osterix expression [25]. Hence, we believe that the β-catenin and HIF-1α could activate the VEGF (HIF-1α target gene) and osterix (Wnt target gene) to crosstalk the canonical Wnt and HIF-1α signaling pathway and then enhance the coupling of osteogenesis and angiogenesis under the costimulation of lithium and copper in BMSCs. However, the mechanism we reported may be just one of the crosstalks between canonical Wnt and HIF-1α signaling pathways in BMSCs. And more researches are needed to explore the interaction between the canonical Wnt, HIF-1α, and other signaling pathways in coupling osteogenesis and angiogenesis.

5. Conclusions

Our study identified that lithium and copper ions' release from the HA/Col scaffold could couple the osteogenesis and angiogenesis in vitro and the Li-Cu-HA/Col scaffold had good mechanical properties and biocompatibility. In addition, we found that the optimal concentration of lithium and copper could effectively activate the canonical Wnt and HIF-1α signaling pathway, respectively. Finally, we indicated that lithium- and copper-mediated osteogenesis and angiogenesis coupling was enhanced through the crosstalk between canonical Wnt and HIF-1α signaling pathways. The results of our study highlight that collaboration of lithium- and copper-doped porous organic-inorganic scaffolds with BMSCs is a possible strategy for the treatment of bone defect.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

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

Acknowledgments

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Supplementary Materials

Supplementary 1. Table S1: primers for RT-qPCR and ChIP.

Supplementary 2. Table S2: western blot, flow cytometry, immunofluorescence, and ChIP antibodies used in this study.

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Research Article

Inhibition of Endoplasmic Reticulum Stress by 4-Phenyl Butyric Acid Presents Therapeutic Effects on Periodontitis: Experimental Studies *In Vitro* and in Rats

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This study investigated the probable mechanisms of endoplasmic reticulum (ER) stress involved in periodontitis *in vitro* and *in vivo*. We isolated periodontal ligament stem cells from periodontitis patients and healthy controls (P-PDLSCs and H-PDLSCs). To further simulate the periodontal microenvironment in patients, lipopolysaccharide (LPS) was used to treat H-PDLSCs. The results showed that periodontitis-related inflammation gave rise to the upregulated expression levels of ER stress representative genes including *GRP78*, *PERK*, *ATF4*, and *CHOP*. In contrast, the treatment of 4-phenyl butyric acid (4-PBA) remarkably suppressed ER stress and supported cell viability. The increased secretion of proinflammatory factors like TNF-α, IL-1β, and IL-6 and the activation of NF-κB pathway were also attenuated by 4-PBA treatment. Moreover, 4-PBA treatment restored the impaired osteogenic differentiation ability of PDLSCs, as demonstrated by the upregulated expression levels of Runx2 and OCN as well as the enhanced Alizarin red staining. Local administration of 4-PBA could rescue alveolar bone resorption of LPS-induced periodontitis rats. Thus, our findings suggested ER stress might act as a promising therapeutic target against periodontitis.

1. Introduction

Periodontitis is among world's most prevalent inflammatory diseases, which has destructive effects on periodontal tissues including gingival, cementum, periodontal ligament, and alveolar bone [1]. It is reported that three quarters of the adult population worldwide have mild periodontal disease (gingivitis) at least and more than one-fifth present with severe and destructive periodontitis [2]. The clinical symptoms include gingival bleeding, formation of periodontal pocket, loss of connective tissue attachment, alveolar bone resorption, and eventually teeth exfoliation. The pathogenesis of periodontitis involves a local inflammatory reaction triggered by the presence of bacterial plaque [3]. Thus, the ultimate objective of periodontal treatment is not simply to control the inflammation, but more importantly, to recover the structure and feature of the damaged tissues in a diseased microenvironment.

Periodontal ligament stem cells (PDLSCs) are a group of heterogeneous mesenchymal stem cells that are located in the periodontal ligament [4]. It is widely accepted that the augmented aberrant differentiation and osteogenic differentiation dysfunction of PDLSCs are closely associated with the alveolar bone resorption during periodontitis [5, 6]. A complex network of signaling pathways governs the differentiation of PDLSCs in periodontitis. Generally, signaling pathways related to cell injury all are considered to be associated to the differentiation of PDLSCs, such as inflammation, autophagy-lysosome pathway, calcium homeostasis, ubiquitin-proteasome system, and endoplasmic reticulum (ER) stress. Generally, the inflammatory microenvironment composed of inflammatory cells and cytokines is noticeable to impede the osteogenic differentiation ability of PDLSCs. Nevertheless, anti-inflammatory therapy still has not achieved the goal to reserve aberrant differentiation of PDLSCs completely, implying there may be other regulatory mechanisms in periodontitis [7, 8]. Our previous studies demonstrated that unfolded protein accumulation in the ER activates the canonical unfolded protein response (UPR) representative genes including *GRP78*, *PERK*, *ATF4*, and *CHOP* and subsequently results in ER stress response, which may facilitate the aggravation of periodontitis [7, 9, 10]. Actually, numerous evidences have documented the intersections between ER stress and inflammation. For example, IREα, a typical transmembrane receptor involved in UPR, could recruit TRAF2 to the ER membrane via the NF-κB pathway to initiate cellular inflammatory responses [11]. However, there is still no available information to provide insight into a communicating network between ER stress and PDLSC fate determination especially osteogenic differentiation in periodontitis.

In the present study, we investigated the probable mechanisms of ER stress in periodontitis *in vitro* and *in vivo*. We found that 4-phenyl butyric acid (4-PBA) confers therapeutic effects on periodontitis by suppressing ER stress and inflammation and restoring PDLSC function. Our findings suggested that targeting ER stress might provide a prospective therapeutic strategy for periodontitis.

2. Materials and Methods

2.1. Cell Culture. We isolated periodontal ligament stem cells from periodontitis patients and healthy controls (P-PDLSCs and H-PDLSCs) as previously described [12, 13]. Briefly, periodontal ligament tissues were isolated from the middle third of the root surface and digested in 3 mg/ml collagenase I (Sigma-Aldrich, St Louis, MO, USA) for 2 h at 37°C. Periodontal ligament stem cells were cultured in 6-well culture dishes (Costar; Corning, NY, USA) in α-minimal essential medium (α-MEM; Gibco, Gaithersburg, MD, USA) supplemented with 0.292 mg/ml L-glutamine (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Sijiqing, Zhejiang, China), 100 mg/ml streptomycin (Invitrogen), and 100 unit/ml penicillin (Invitrogen) in 5% CO_2 at 37°C. Limiting dilution technique purified the stem cells into single cell colony cultures and then pooled and expanded. Cells from passages 2-5 were used in the following experiments. An identified ER stress inhibitor, 4-phenyl butyric acid (4-PBA, 5 mM; Sigma-Aldrich) was used to treat cells for 24 h, and lipopolysaccharide (LPS, 10 μg/ml) was acquired from Pepro-Tech (Pepro-Tech, NJ, USA).

Table 1: Primer sequence used for RT-qPCR.

Gene	Primer	Sequence 5′ to 3′
PERK	Forward	GTGATAAAGGTTTCGGTTGCTG
	Reverse	TGTTTTCTGTGGCTCCTCTGG
GRP78	Forward	TCAAGTTCTTGCCGTTCAAGG
	Reverse	AAATAAGCCTCAGCGGTTTCTT
ATF4	Forward	CATGGGTTCTCCAGCGACA
	Reverse	TCTGGCATGGTTTCCAGGTC
CHOP	Forward	CAAGAGGTCCTGTCTTCAGATGA
	Reverse	TCTGTTTCCGTTTCCTGGTTC
Runx2	Forward	CCCGTGGCCTTCAAGGT
	Reverse	CGTTACCCGCCATGACAGTA
OCN	Forward	CCCAGGCGCTACCTGTATCAA
	Reverse	GGTCAGCCAACTCGTCACAGTC
GAPDH	Forward	CTGCAAGAACAGCATTGCAT
	Reverse	GACCACCTGGTCCTCAGTGT

2.2. Real-Time Quantitative PCR. By using the SYBR Premix Ex Taq II kit (Takara, Shiga, Japan), RT-qPCR reactions were performed by using CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). We used standard settings: 94°C, 3 min; 94°C, 15 s; 60°C, 30 s; repeated 40 cycles, and then dissociation. Each assay ran in triplicate. The relative standard curve method calculated the arbitrary mRNA concentrations. The $^{\Delta\Delta}$-ct method normalized to GAPDH. The primer sequences used in the present study are showed in Table 1.

2.3. Western Blot Analysis. Western blot analysis was performed as described in our previous studies [7, 9, 10]. RIPA lysis buffer extracted proteins, and then, G250 protein assay (Beyotime, Jiangsu, China) extracted soluble protein. Protein samples were loaded on 10% sodium dodecyl sulfate polyacrylamide gels (Invitrogen), transferred to polyvinylidene fluoride membranes (Bio-Rad), and blocked with 5% nonfat milk powder. Membranes were incubated overnight with the following primary antibodies for human Runx2 (Cell Signaling, Beverly, MA, USA), OCN (Santa Cruz, Dallas, TX, USA), NF-κB (Santa Cruz), p-NF-κB (Santa Cruz), and β-actin (Cowin Biotech, Beijing, China). Torseradish peroxidase- (HRP-) conjugated secondary antibody (Cowin Biotech) incubated the membranes for 2 h at room temperature. Protein signals were visualized by using the ECL Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA).

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). The samples were centrifuged at for 5 min 3000 g after a 15 min elution phase at room temperature in Eppendorf tube with 50 ml of PBS (Sigma-Aldrich). The supernatants were stored at -80°C until used. The concentrations of proinflammatory

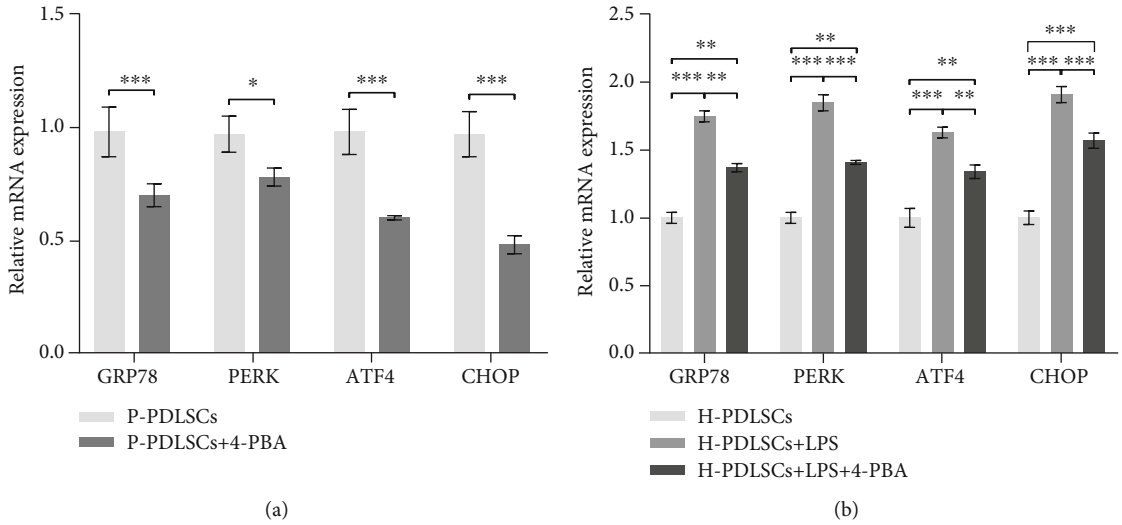

Figure 1: 4-PBA reverses ER stress in PDLSCs under inflammatory periodontitis condition. RT-qPCR showed (a) the expression levels of UPR representative genes *GRP78*, *PERK*, *ATF4*, and *CHOP* in P-PDLSCs treated with or without 5 mM 4-PBA for 7 days; (b) the expression levels of UPR representative genes were measured in H-PDLSCs treated in the absence or presence of 10 mg/l LPS or LPS in association with 5 mM 4-PBA for 7 days. The consequences were normalized to the *GAPDH* gene, and data represent mean ± standard deviations ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

factors TNF-α, IL-1β, and IL-6 levels were assayed with commercial ELISA kits according to the manufacturer's instructions (BioSource, Camarillo, Calif., USA) in duplicate.

2.5. CCK-8 Assay. A total of 1×10^3 cells of 0.1 ml *per* well were cultured in a 96-well plate in 10% FBS in three replicate wells. After drug treatment, 10 μl of CCK-8 kit reagent (Beyotime) was added to 100 μl of cell culture medium and incubated for 1.5 h at 37°C. The absorbance of each well at 450 nm was measured by using a microplate reader (Bio-Rad).

2.6. Osteogenic Differentiation In Vitro. The medium was changed to 2 mol/l β-glycerophosphate, 10 nmol/l dexamethasone, and 100 μg/ml ascorbic acid (Sigma-Aldrich). RNA was extracted for 1 week for the osteogenic genes by using RT-qPCR analysis. Protein was extracted after 14 days for osteogenic markers by using Western blot analysis. Osteoblast calcium deposits were determined by staining with 1% Alizarin Red (Sigma-Aldrich) as previously described [14]. 1% cetylpyridinium chloride (Sigma-Aldrich) dissolved the mineralized nodules, and a spectrophotometer (Epoch, Bio-Tek, USA) measured the OD value at 562 nm. Total protein quantity of each sample normalized the results to exclude the impact of cell numbers.

2.7. Drug Administration in Sprague–Dawley (SD) Rats of Experimental Periodontitis. A periodontitis model was established as described before [15]. We distributed three groups of nine eight-week-old SD rats: saline; LPS (10 μg/day) and LPS (10 μg/day) +4-PBA (5 nmol/day) with three rats *per* group. Then, the drug was injected between the first and second upper molars into the maxillary palatal gingiva in each group and repeated every two days for 7 days. The rats were anesthetized and euthanized by exsanguination. The SD rats' whole heads were removed, and a micro-CT system (Siemens Inveon Micro-CT, Munich, Germany) scanned and analyzed the maxillaries. The alveolar bone height of four different sites was recorded by measuring the length from the alveolar bone crest to the cemento-enamel junction (CEJ) in two molars. Body weight, heart/body weight, liver/body weight, spleen/body weight, adrenal gland/body weight, and kidney/-body weight were measured to appraise the effect of drug administration on general condition.

2.8. Statistical Analysis. Data are represented as means ± standard deviations of each independent experiment ($n = 3$). Comparisons between the two groups were calculated by independent two-tailed unpaired Student's t-test and among numerous comparisons by the Bonferroni correction of analysis of variance in the GraphPad Prism software (San Diego, CA, USA). Significant differences were considered when $P < 0\,05$.

3. Results

3.1. 4-PBA Reverses ER Stress in PDLSCs under Inflammatory Periodontitis Condition. Our previous study has identified the upregulation of the classical UPR genes including *GRP78*, *PERK*, *ATF4*, and *CHOP* in primary P-PDLSCs [7]. To testify the effect of 4-PBA on ER stress in the inflammatory microenvironment, we treated P-PDLSCs with 4-PBA (5 mM) for 7 days. As shown in Figure 1(a), the UPR target genes in P-PDLSCs were significantly downregulated. To further simulate the periodontal microenvironment in patients, LPS was used to treat H-PDLSCs. The consequences showed that 4-PBA could significantly decrease the levels of URP target genes in LPS-treated H-PDLSCs. Nevertheless, their expression levels were still expressively higher than those in H-PDLSCs (Figure 1(b)).

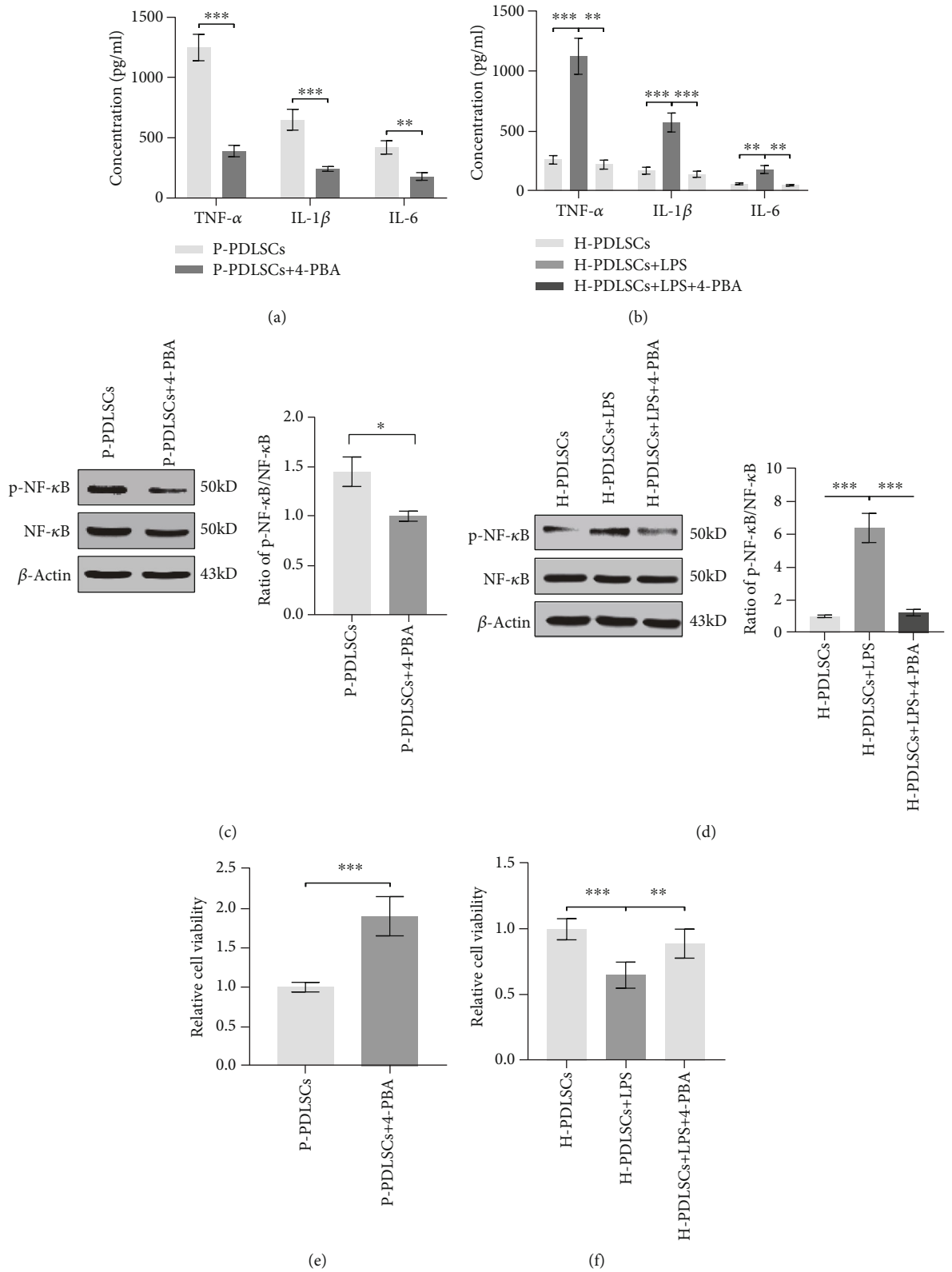

Figure 2: 4-PBA attenuates inflammatory response of P-PDLSCs and LPS-treated H-PDLSCs. The secretion of proinflammatory factors TNF-α, IL-1β, and IL-6 was detected by using ELISA in (a) P-PDLSCs treated without or with 5 mM 4-PBA and (b) H-PDLSCs treated in the absence or presence of 10 mg/l LPS or LPS in association with 5 mM 4-PBA. Western blot analysis showed the expression levels of p-NF-κB and NF-κB, as well as the ratio of p-NF-κB/NF-κB in (c) P-PDLSCs treated without or with 5 mM 4-PBA or in (d) H-PDLSCs treated in the absence or presence of 10 mg/l LPS or LPS in association with 5 mM 4-PBA. Internal control used β-actin. (e, f) Cell viability was determined by using CCK-8 assay after two days treatment. The data represent mean ± standard deviations ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

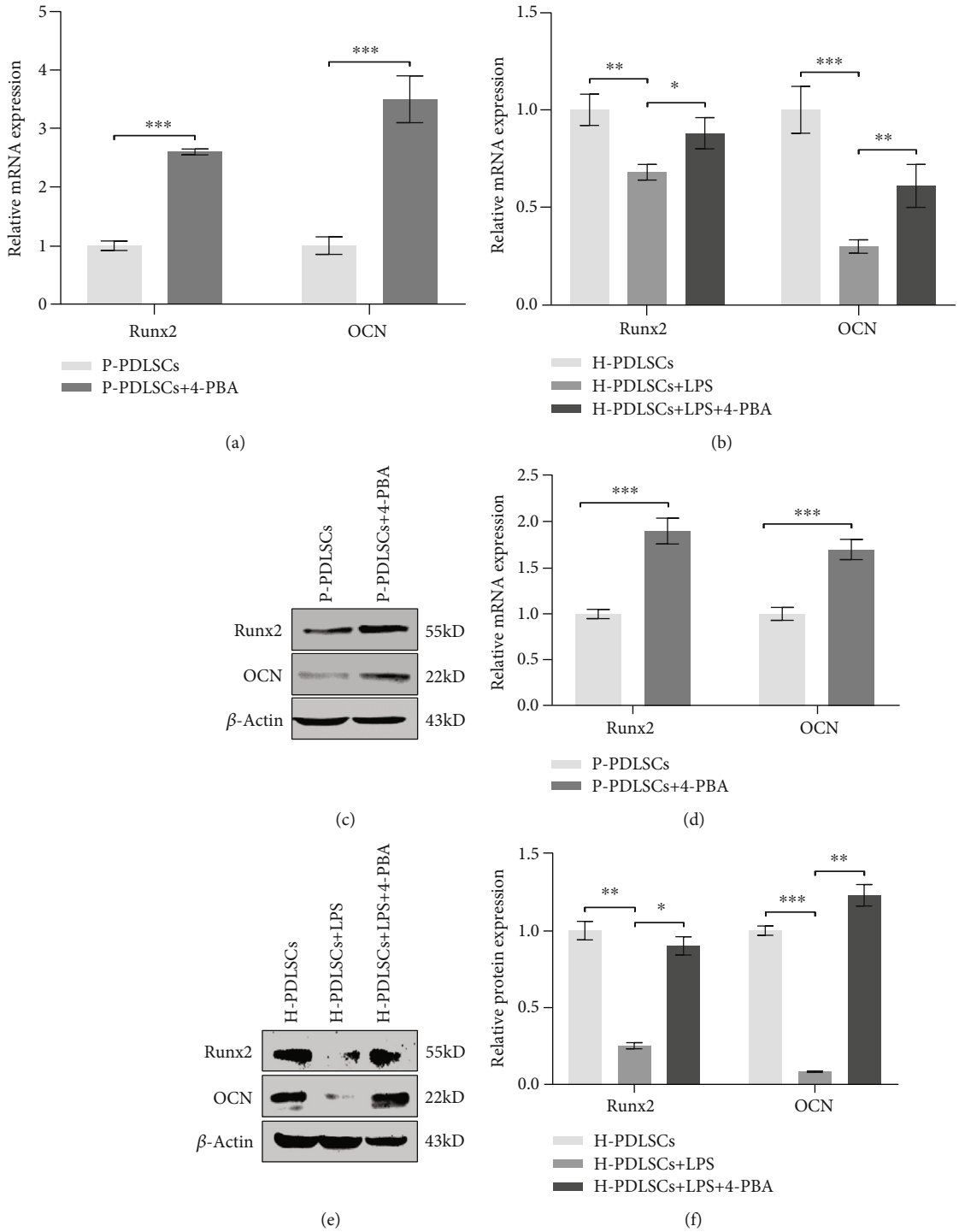

Figure 3: 4-PBA restores the impaired osteogenic differentiation ability of PDLSCs in the inflammatory microenvironment. The mRNA and protein expression levels of Runx2 and OCN were determined by RT-qPCR after one week (a, b) and Western blot analysist after 14 days (c–f). P-PDLSCs were treated with or without 5 mM 4-PBA, and H-PDLSCs treated in the absence or presence of 10 mg/l LPS or LPS in association with 5 mM 4-PBA. The consequences were normalized to the *GAPDH* gene, and data represent mean ± standard deviations ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2. 4-PBA Attenuates Inflammatory Response of P-PDLSCs and LPS-Treated H-PDLSCs. Next, we assessed the expressions of proinflammatory cytokines in P-PDLSCs and LPS-treated H-PDLSCs. ELISA showed that treating with 4-PBA could significantly decrease the secretion of proinflammatory factors TNF-α, IL-1β, and IL-6 in P-PDLSCs and LPS-treated H-PDLSCs (Figures 2(a) and 2(b)). To explore the underlying mechanism, Western blot analysis was used to determine the expressions of p-NF-κB and NF-κB, and the results showed that treatment of 4-PBA could remarkably reduce

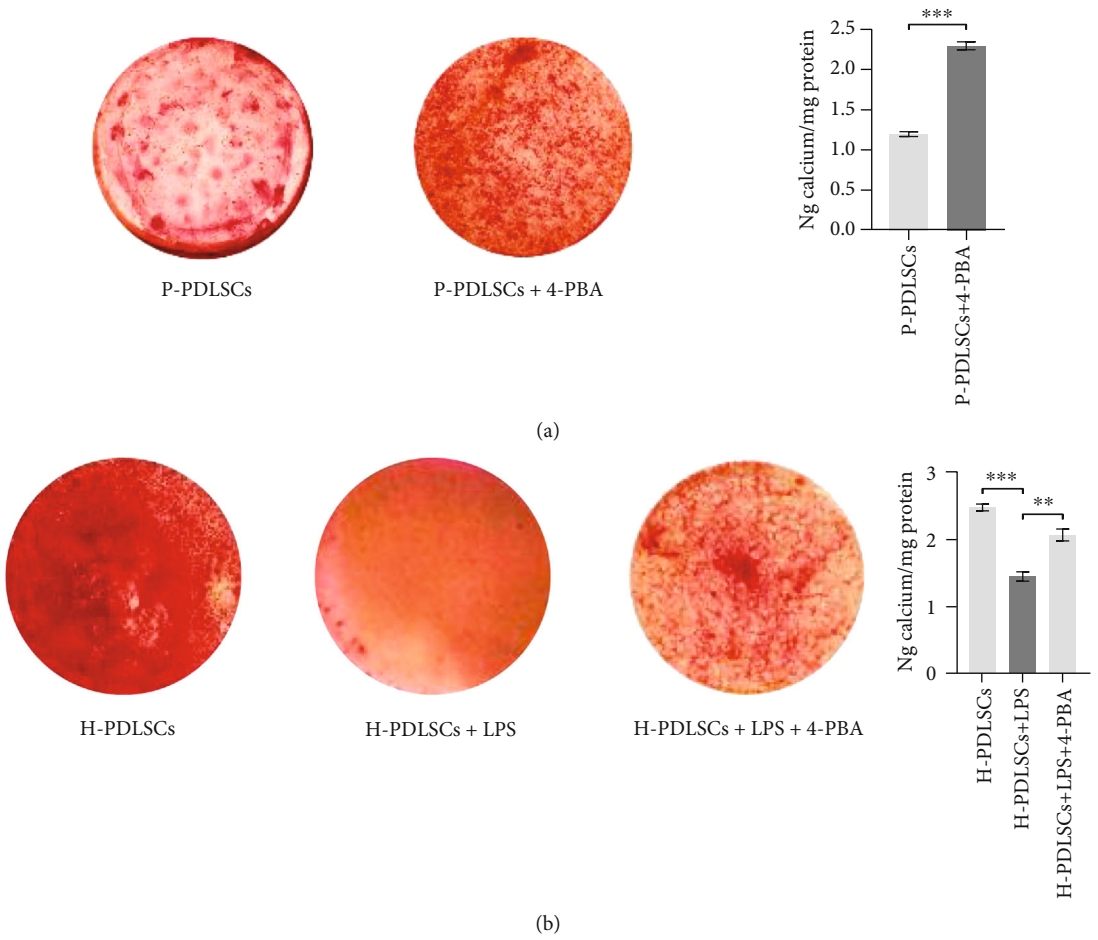

Figure 4: Alizarin red staining. (a) P-PDLSCs were treated without or with 5 mM 4-PBA, and (b) H-PDLSCs treated in the absence or presence of 10 mg/l LPS or LPS in association with 5 mM 4-PBA for 28 days. The data represent mean ± standard deviations ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the p-NF-κB/NF-κB ratio (Figures 2(c) and 2(d)). Further, the results of CCK-8 indicated that 4-PBA could support PDLSC viability in the inflammatory microenvironment (Figures 2(e) and 2(f)).

3.3. 4-PBA Restores the Impaired Osteogenic Differentiation Ability of PDLSCs in the Inflammatory Microenvironment. As shown in Figure 3, RT-qPCR (Figures 3(a) and 3(b)) and Western blot results (Figures 3(c)–3(f)) showed that the osteogenic differentiation associated biomarker proteins, like Runx2 and OCN, was significantly upregulated after 4-PBA treatment in both P-PDLSCs and LPS-treated H-PDLSCs. In addition, Alizarin red staining demonstrated that 4-PBA-treated cells formed more mineralization nodules in PDLSCs in the inflammatory microenvironment (Figures 4(a) and 4(b)).

3.4. Local Administration of 4-PBA Rescues Alveolar Bone Resorption in an LPS-Induced Periodontitis Rat Model. To further examine the effect of 4-PBA on periodontitis *in vivo*, LPS administration was recruited to establish a periodontitis rat model. As shown in Figure 5, the LPS group showed more excessive bone resorption as the farthest distance, from the alveolar bone crest to the CEJ, was observed in all the six sites. Whereas in the LPS+4-PBA group, the alveolar bone resorption of three molars was notably lessened, indicating that 4-PBA could rescue alveolar bone resorption in periodontitis. On the other hand, drug administration including LPS and LPS+4-PBA had little effect on the general condition of control rats (Table 2). Body weight, heart/body weight, spleen/body weight, adrenal gland/body weight, and kidney/body weight had little difference between the three groups, while liver/body weight was decreased in the LPS group compared to the control group and the LPS+4-PBA group (Table 2).

4. Discussion

In the present study, we provided a communicating network between ER stress and PDLSC function in periodontitis-related inflammation. To this end, P-PDLSCs and H-PDLSCs used as model cells and 4-PBA, an identified ER stress inhibitor, were used in this study. To simulate inflammatory periodontitis condition *in vitro* and *in vivo*, LPS was used to treat H-PDLSCs and also locally administrated in rats. The results showed that 4-PBA could downregulate

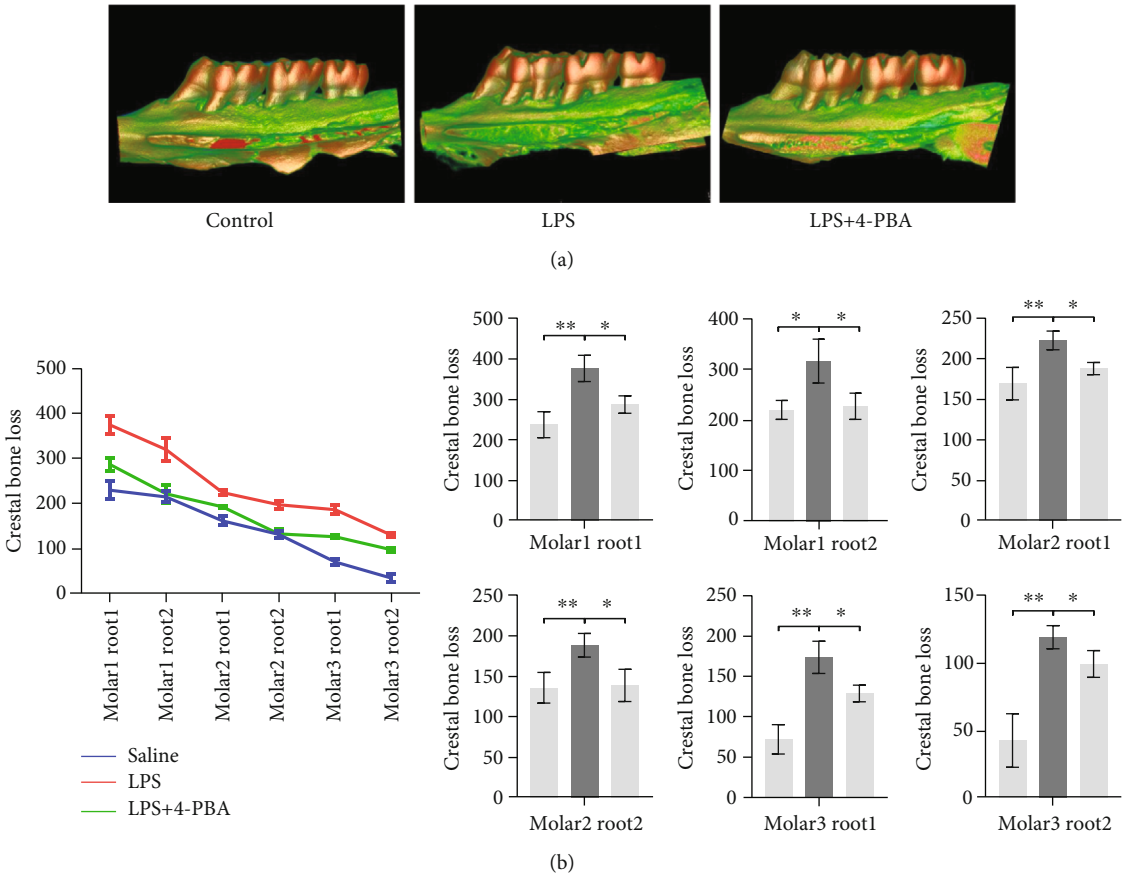

Figure 5: Local administration of 4-PBA rescues alveolar bone resorption in an LPS-induced periodontitis rat model. (a) Micro-CT acquired the representative images of the alveolar bone loss. (b) Alveolar bone resorption analysis of maxillary molars. Six sites for three molars were analyzed morphometrically (one site for each root of one tooth). The distance between the cement-enamel junction (CEJ) and alveolar crest of the mesial and distal roots of the three maxillary molars was assessed under a stereoscopic microscope. The results indicated more alveolar bone loss in the LPS (10 μg/day) treated groups compared to the saline group (10 μl). Local administration of 4-PBA (5 nmol/day) rescued the bone resorption induced by LPS. The data represent mean ± standard deviations ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 2: Effect of 4-PBA local administration on rats with experimental periodontitis. The body weight, heart/body weight, liver/body weight, spleen/body weight, adrenal gland/body weight, and kidney/body weight were analyzed in the saline group, LPS group, and LPS in combination with the 4-PBA group. The data represent mean ± standard deviations ($n = 3$). $^{*}P < 0.05$.

	Saline ($\times 10^{-3}$kg)	LPS ($\times 10^{-3}$kg)	LPS+4PBA ($\times 10^{-3}$kg)
Body weight	0.36 ± 0.2	0.32 ± 0.1	0.33 ± 0.05
Heart/body weight	3.08 ± 0.52	3.38 ± 0.31	2.67 ± 0.36
Liver/body weight	47.37 ± 3.31	34.09 ± 2.65*	39.33 ± 7.3
Spleen/body weight	2.69 ± 0.49	2.47 ± 0.13	2.6 ± 0.82
Adrenal gland/body weight	0.21 ± 0.08	0.20 ± 0.1	0.26 ± 0.06
Kidney/body weight	8.10 ± 0.29	7.98 ± 0.86	7.87 ± 1.31

the expression levels of ER stress-associated genes *GRP78*, *PERK*, *ATF4*, and *CHOP* in periodontitis-related inflammation. Besides, declined proinflammatory factors TNF-α, IL-1β, and IL-6 and suppression of NF-κB signaling pathway after 4-PBA treatment indicated inhibited cellular inflammatory responses. Subsequently, upregulated Runx2 and OCN expression detected by RT-qPCR and Western blot analyses and enhanced Alizarin red staining suggested the restored osteogenic differentiation capability of PDLSCs in the inflammatory microenvironment. In addition, local administration of 4-PBA rescued alveolar bone resorption of LPS-induced periodontitis rats. Thus, our findings indicated 4-PBA exhibited a therapeutic potential against periodontitis both *in vitro* and *in vivo*.

Periodontitis is a characteristic inflammatory disease which is the main cause of periodontal tissue destruction

and potentially tooth loss. Periodontal ligament stem cells are the core examinee stem cells for periodontal regeneration. Due to their self-renewal potential and multidifferentiation capability, PDLSCs have been widely used in tissue regeneration including periodontium [16]. The function and fate of PDLSCs are closely associated with the pathogenesis, progression, treatment, and prognosis in periodontitis [17, 18]. However, PDLSCs from periodontitis patients show deficient osteogenic differentiation ability in comparison with cells from healthy individuals [19]. Interestingly, osteogenic differentiation deficiency could not be retrieved after *ex vivo* culture and expansion [20]. In addition to chronic inflammation, the osteogenic capacity of PDLSCs might be affected by other factors. ER stress, caused by UPR, is an essential strategy in response to the drastic changes of the extracellular environment, which has been demonstrated to be involved in multiple oral diseases [7, 21, 22]. As previous studies reported, the IRE1α branch of the UPR might be associated with the secretion of proinflammatory cytokines in many kinds of cells such as endothelial cells and macrophages [23]. Endoplasmic reticulum stress impels macrophages to induce mature IL-1β in response to TLR4 stimulation via a TRIF- and caspase-8-dependent pathway [24]. In the present study, we found the upregulated expression levels of ER stress representative genes *GRP78*, *PERK*, *ATF4*, and *CHOP* both in P-PDLSCs and LPS-treated H-PDLSCs. These results indicated ER stress is a crucial pathogenesis in periodontitis. Moreover, periodontitis-related inflammation in the microenvironment resulted in lower cell viability, secretion of proinflammatory factors, and activation of NF-κB pathway. In accordance with our previous studies [7, 21, 22], periodontitis impaired ER function and causes ER stress, which in turn results in deficient osteogenic differentiation of PDLSCs.

To investigate functional significance of ER stress in periodontitis, an identified ER stress inhibitor 4-PBA was used here. Previous studies have showed the therapeutic potential of 4-PBA for many kinds of human diseases [25–27]. Mechanically, 4-PBA could repress the overactivated ER stress and attenuate the UPR by stabilizing protein conformation, enhancing folding capacity of ER, and facilitating trafficking of mutant proteins [28–31]. In the present study, we found that the 4-PBA treatment remarkably suppressed ER stress and supported cell viability. The treatment of 4-PBA also reduced the enhanced secretion of proinflammatory cytokines by suppressing the activation of NF-κB pathway. Indeed, previous studies have demonstrated that ER stress has been related to the activation of NF-κB, a crucial transcription factor for many inflammatory processes [32]. We demonstrated that 4-PBA could interfere with the inflammation-related pathways in periodontitis, which might provide a new insight into the therapeutic mechanism of 4-PBA. Furthermore, 4-PBA treatment restored the impaired osteogenic differentiation ability of PDLSCs in the inflammatory microenvironment, and local administration of 4-PBA could rescue alveolar bone resorption of LPS-induced periodontitis rats. Our previous studies have shown that ER stress negatively affects the osteogenic differentiation function of PDLSCs via PERK and IRE1α pathways [7, 8]. Indeed, accumulating experimental evidence suggested the harnessing of ER stress has proven to be a particularly plausible therapeutic strategy for regulating bone metabolism and tissue repair, such as osteoarthritis [33], intervertebral disc degeneration [34], osteogenesis imperfecta [35, 36], and implant osseointegration [37]. It is noted that drug administration of LPS or LPS+4-PBA had adverse effects on the general condition of rats with lower liver/body weight than that of the control group, which may result from the hepatotoxicity of LPS [38].

Although our findings demonstrated that 4-PBA could suppress inflammation and restore the impaired osteogenic differentiation ability of PDLSCs by affecting the NF-κB pathway, other molecular mechanisms have not been further studied. We also did not use multiple models of periodontitis, such as bacteria, ligature, or hypoxia, to validate these findings. These deficiencies will be explored in depth in future studies.

5. Conclusion

The present study investigated the possible mechanism ER stress associated with periodontitis. We investigated that inhibition of endoplasmic reticulum stress by 4-PBA suppressed ER stress and inflammation, supported cell viability, and restored the defective osteogenic differentiation of PDLSCs under inflammatory periodontitis conditions. Local administration of 4-PBA rescued alveolar bone resorption in an LPS-induced periodontitis rat model. Our findings suggested the potential of harnessing ER stress in order to develop a novel therapeutic approach for periodontitis.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors declare that no competing interests exist.

Authors' Contributions

Yang Feng, Rong Zhang, and Yi-rong Wang contributed equally to this work. Peng Xue, Yang Jiao, Yang Feng, and Rong Zhang designed, executed the experiments, and drafted the manuscript. Yang Feng, Yi-rong Wang, and Peng Xue collected and analyzed the data. Fei Chen, Qiang Luo, and Chuan Cai fabricated specimens. Yang Jiao and Peng Xue revised the manuscript. All the authors approved the manuscript. Yang Feng, Rong Zhang, and Yi-rong Wang contributed equally to this work and should be considered as co-first authors.

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Review Article

Myogenic Differentiation of Stem Cells for Skeletal Muscle Regeneration

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Stem cells have become a hot research topic in the field of regenerative medicine due to their self-renewal and differentiation capabilities. Skeletal muscle tissue is one of the most important tissues in the human body, and it is difficult to recover when severely damaged. However, conventional treatment methods can cause great pain to patients. Stem cell-based tissue engineering can repair skeletal muscle to the greatest extent with little damage. Therefore, the application of stem cells to skeletal muscle regeneration is very promising. In this review, we discuss scaffolds and stem cells for skeletal muscle regeneration and put forward our ideas for future development.

1. Introduction

Skeletal muscle accounts for 30-40% of the weight of a healthy human body and is necessary for free movement of the human body [1]. Muscle regeneration relies on a group of small adult stem cells called satellite cells. Satellite cells are quiescent under resting conditions, but they can quickly reenter the cell cycle after being injured or receiving growth signals. Activated satellite cells will migrate and proliferate extensively for muscle regeneration [2]. Though healthy skeletal muscle has promising recovery ability to cope with minor injuries in daily life, the fate of satellite cells is strictly controlled by internal and external factors. This fragile balance may be disturbed by aging, hereditary myopathy, and massive muscle loss. Surgery is commonly used in clinical practice, but the high cost and considerable may discourage some patients. Under this circumstance, tissue engineering (TE) attracts the attention of researchers and has become the new tool to treat skeletal muscle diseases and promote skeletal muscle regeneration [3–5].

The three pillars of tissue engineering are cells, biomaterials, and environment [6]. Stem cells are undifferentiated cells that are presented in all parts of the body, which possesses the ability of self-renewal and differentiation. Since a number of stem cells have been proven to have the potential for myogenic differentiation, stem cells are considered the most potential cell source of skeletal muscle TE [7–9]. As one of the three elements of tissue engineering, scaffolds play an important role in the whole technology. The function of scaffolds is to simulate extracellular matrix. Because different tissues have their unique extracellular matrix, different types of scaffolds can steer cell differentiation towards different directions.

In this review, we describe the directional differentiation of skeletal muscle cells according to the sequence of three pillars of tissue engineering. We first introduced the process of skeletal muscle regeneration under physiological conditions and the chemical signals expressed at each differentiation stage. Second, we introduced some biomaterials and environmental factors currently used for myogenic differentiation. Third, we listed several commonly used stem cells for myo-

genic differentiation and described the recent advances in directing into skeletal muscle. Finally, we put forward our own views on myogenic differentiation and make an outlook on its future use.

2. Muscle Regeneration

2.1. Myogenic Markers. In adult muscles, satellite cells are usually mitotically quiescent. In general, once exposed to signals from the damaged environment, satellite cells will leave their quiescent state, reenter the cell cycle, and start proliferating (satellite cell activation). Some daughter cells continue to differentiate, while others return to quiescence to replenish the reserve population of satellite cells, then, the activated satellite cells form multinucleated myotubes after mitosis [10, 11]. Many pathological conditions, such as muscular dystrophies (MDs) or muscle wasting, cannot provide sufficient signals for satellite cells, which will impair their regeneration potential [2]. The multistep muscle formation process is strictly controlled by a complex gene regulatory network [12]. First, many miRNAs (microRNAs) are small noncoding RNA molecules that target mRNA, are used to fine-tune gene expression, and are also an important part of the network [13, 14]. Second, muscle satellite cells can be characterized by a combination of several genetic markers, including paired box proteins, Pax7 (considered a clear MUSCs marker), and muscle regulatory factors MRFs, including MYF5, MYOD, MYOG (myogenin), and MRF4 [15–17]. PAX7 is a paired homeobox transcription factor, which specifies the myogenic properties of muscle stem cells and acts as a nodal factor by stimulating proliferation and inhibiting differentiation [18]. Some studies have shown that after Pax7 is missing, satellite cells and myoblasts show cell cycle arrest and imbalance of myogenic regulatory factors. In a word, Pax7 is an absolute requirement for the function of adult skeletal muscle satellite cells [19]. Third, myogenesis depends on the precise and dynamic integration of multiple Wnt signals, this allows the self-renewal and progress transcription factors such as myogenic factor 5 (MYF5), and myogenic differentiation factor 1 (MYOD) can be specifically expressed in myogenic cells, but not expressed in stationary satellite cells [2]. On the one hand, MYOD is a key transcription factor for myogenesis. On the other hand, the inactivation of MRF4 will cause subtle changes in muscle strength and innervation [20]. Researchers have shown that adult satellite cells originated from progenitor cells that first expressed the myogenic assay gene MYF5 in the fetal stage of myogenesis [21] (see Figure 1). In addition, during embryonic development, Wnt signals control the expression of myogenic regulatory factors (MRFs), and MRFs are essential for the development of myogenic lineages [22]. Above all, the formation of skeletal muscle is a process strictly regulated of muscle precursors in the myogenic lineage [23]. It is worth noting that the resting state of satellite cells has some similarities with hibernation, in which the cells are kept in a minimum energy state. The energy needed comes from the catabolism of storing large molecules and can minimize energy consumption, thereby keeping cells at the forefront of cell and developmental biology [24].

2.2. MRF in Differentiation Stages. Muscle regeneration can be divided into several stages, which are characterized by different expression of myogenic regulatory factors (MRFs). In the stationary phase, satellite cells are not active, but ready to activate. In a way, quiescent satellite cells usually express markers such as Pax7 and Myf5. After muscle injury, satellite cells are stimulated by various signals generated by the injury environment and finally differentiate satellite cells and migrate to the injured site, then reenter the cell cycle to proliferate. At this stage, they are called myoblasts and express the myoblast marker Pax7, MYF5, and/or MYOD. After the proliferation phase, myoblasts exit the cell cycle and differentiate into mature muscle cells [2, 20, 21]. About 80% of Pax7+ cells express MYF5, but after activation and proliferation, the expression of Pax7 and MYF5 decreases, while MYOD increases correspondingly in the proliferative phase [20, 21, 25].

2.3. Advanced Studies Involved Muscle Regeneration. Researchers extracted extracellular matrix (ECM) from the thigh muscles of adult rats and presented it to the cells as a surface coating. They deserved that compared with standard growth noodles, myogenic cells cultured on ECM extracts have stronger proliferation and differentiation capabilities. It is confirmed that ECM molecules extracted from skeletal muscle can positively affect the proliferation and differentiation of satellite cells and myoblasts [26]. Rayagiri et al. found that skeletal satellite cells induced local remodeling of ECM and the deposition of laminin-α1 and laminin α5 into the basal layer of the satellite cell niche. Genetic ablation of laminin-α1, destruction of integrin-α6 signal, or destruction of matrix metalloproteinase activity can impair the expansion and self-renewal of satellite cells; it is proved that the remodeling of ECM is an essential process for stem cell activity to support reproduction and self-renewal [27]. Another researcher has proved that the presence of adipose tissue-derived stromal cells (ADSCs) derived from adipose tissue can promote skeletal muscle regeneration, and this effect can be enhanced by pretreatment of IL-4 and SDF-1 cells [28]. On the other hand, mesenchymal progenitors (MPs) are also involved in regeneration. Scott et al. determined that methylation in cancer 1 (Hic1) is a marker of skeletal muscle MP, and it further shows that the loss of Hic1 leads to the proliferation of MP. These suggest that Hic1 + MP coordinates many aspects of skeletal muscle regeneration by providing stage-specific immune regulation and nutritional and mechanical support. They further show that they have unique functions and genealogical potential. It can be concluded that HIC1 regulates MP quiescence and identifies MP subgroups with short-term and long-lasting effects in muscle regeneration [29].

2.4. Scaffolds in Tissue Engineering

2.4.1. Cell Culture: Transition from 2D to 3D. The method of two-dimensional (2D) cell culture is the basic method of cell culture. It first appeared in the early 20th century [30], which has existed for many years as the most extensive and conventional culture method of cells and plays an important role in stem cell research, biomedical fields, and so forth [31, 32].

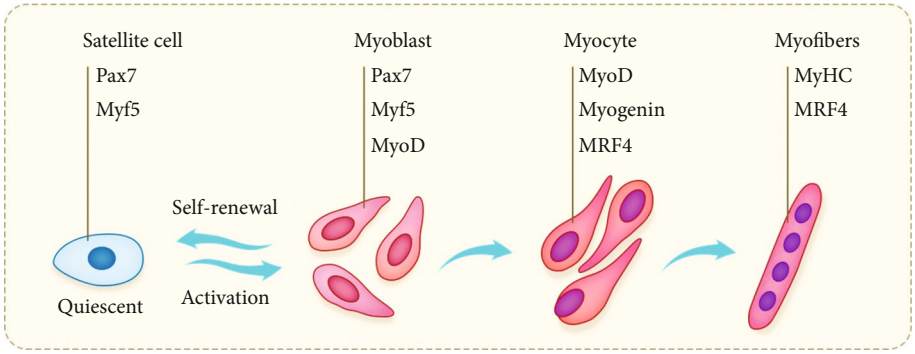

Figure 1: Schematic diagram of muscle regeneration. Skeletal muscle tissue regeneration is regulated by a genetic cascade involving Pax7 and MRFs, which drive every step of satellite cell activation, transient expansion of progenitor cells, and the differentiation and formation of new muscle fibers. Interestingly, satellite cell self-renewal can retain a small number of rested cells after regeneration to meet future regeneration needs.

However, this classical method was born with obvious imperfections [33, 34], because all of the cells in the human body are in a complex three-dimensional environment, and the cells cultured in 2D mode lack interaction with adjacent cells and extracellular matrix, resulting in cell signal imbalance and cell morphological changes [35]. In recent years, three-dimensional (3D) culture technology has gradually become one of the hot research fields in cell biology and tissue engineering (see Figure 2). The three elements of tissue engineering are seed cells, scaffolds, and growth factors [36, 37]. The cells cultured in 3D showed different characteristics from those in 2D. Therefore, it is in the foreseeable future that 2D cell culture gradually withdrew from the stage of history and was replaced by more perfect 3D cell culture technology. 3D cell culture technology has obvious advantages, but it will be a long process to completely replace 2D cell culture technology because 3D cell culture absolutely requires more funds, complex operation, and experience.

2.4.2. A Brief Introduction of Scaffolds and Their Application Examples for Muscle Regeneration. The utilization of scaffolds is an indispensable part of tissue engineering, a useful technique for muscle regeneration, which can provide temporary mechanical support and necessary growth environment for seed cell adhesion, growth, proliferation, and differentiation [37]. Scaffolds are defined as three-dimensional (3D) solid biomaterials that play an indispensable role in tissue regeneration [35, 38]. The physical and chemical properties of scaffolds play an important role in three-dimensional cell culture, which always determines the fate of cells or the outcome of implantation. It is necessary to control these properties for various tissue engineering applications. According to the source, scaffold materials can be divided into natural materials, synthetic materials, and composite materials. The function of scaffolds in tissue engineering is to mimic the function of ECM [5]. ECM is unique in specific tissue whose properties are required for 3D scaffolds in engineering different tissue [39]. We should take many aspects into consideration when selecting scaffold: architecture, cell and tissue compatibility, and bioactivity and mechanical properties. Four main scaffold methods for tissue engineering have been developed rapidly including: premade porous scaffolds for cell seeding, 、decellularized ECM, cell sheets with self-secreted ECM, and cell encapsulation in self-assembled hydrogel matrix [36].

The past few decades have witnessed the development of applying tissue engineering techniques to muscle regeneration. Scaffolds used to support skeletal muscle regeneration should accommodate and promote the formation of densely packed, highly-aligned myofibers throughout a large tissue volume [5]. Scaffolds used for muscle regeneration should carry polarity-oriented property to maintain the parallel differentiation and growth of multinucleated myotubes. In addition, tension and elasticity are required to ensure the contractile function of myotubes. In the 2D level, well-arranged murine skeletal myoblasts (C2C12) cells adhered to bilayer sheets through using nanoribbons can promote their differentiation into mature myotubes and help express myogenic genes [40]. Electroconductive nanosubstrates can enhance myogenic differentiation and maturation [41]. However, the 2D culture model might lose the tissue architecture developed during tissue culture, and the number of sheets that can be stacked has an upper limit (i.e., limited thickness) since cells cannot secure nutrients from a distance (e.g., ~150 μ m) which otherwise causes necrosis [42]. When it comes to 3D level, among a variety of scaffold materials, materials with anisotropic architectures, in possession of high similarity in morphology and function to the native tissue, could be an excellent selection to apply to muscle tissue engineering [42]. The well-aligned orientation of muscle tissue, with parallel bundles of muscle fibers, is a guarantee for performing its systolic and diastolic functions. Takahashi et al. [43] has proven that to form an anisotropic myoblast sheets was exactly able to contribute to self-organization behavior and well organize the 3D orientation of myoblasts and myotubes. Chen et al. [44] utilized collagen scaffolds with concave microgrooves to mimic muscle basement membrane and finally found that myoblasts in the engineered muscle tissue highly expressed myosin heavy chain and synthesis of muscle ECM regardless of different groove sizes. To mimic native skeletal muscle tissue, Wang et al. [45] generated hydrogel core-shell scaffolds combining with nanofiber yarns core and successfully induced alignment, elongation, and differentiation of C2C12. Aligned nanofibrous cylinders

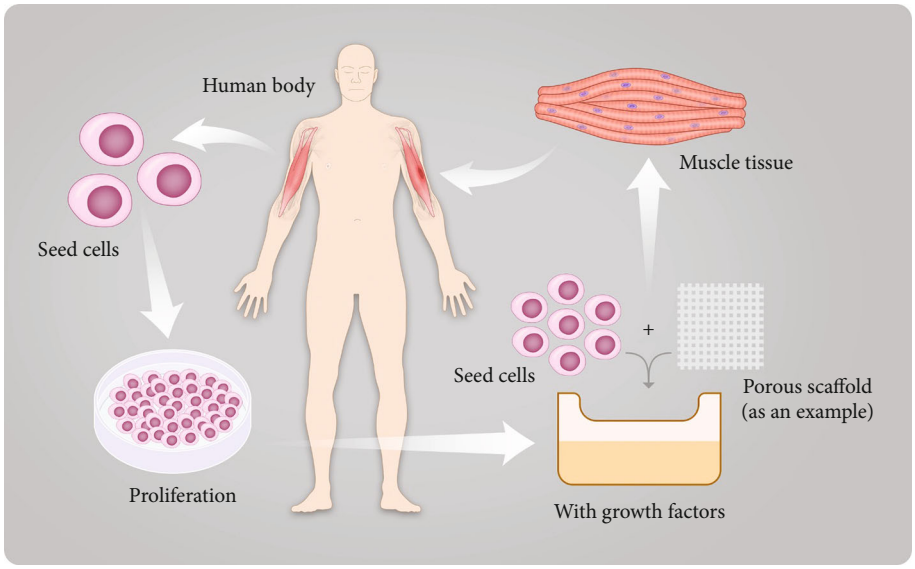

Figure 2: Schematic diagram of the general process of skeletal muscle tissue engineering. Taking the method of treating the biceps brachii defect with porous scaffolds as an example. First, seed cells are obtained from the biceps brachii on the healthy arm and are cultured in vitro. Next, we should make seed cells attached to porous scaffolds and add growth factors. Finally, a small amount of healthy skeletal muscle tissue is obtained and then implanted into the human body.

as scaffolds could be chosen to form aligned, densely populated myotubes, even without a substrate support [46]. Plus, Ku et al. [47] fabricated nanofiber scaffolds with electrical conductivity property and confirmed there is a synergic effect of them in the midst of stimulating muscle cell differentiation. Choi et al. [48] also performed a similar investigation. For volumetric muscle loss (VML) injury, porous collagen-GAG scaffolds implantation could be adopted as a possible good and plausible treatment option to increase muscle hypertrophy and restore functional capacity [49]. In addition to exploiting the chemical or physical attributes of scaffolds, researches of biologic scaffolds for muscle regeneration have recently emerged [50]. Qiu et al. [51] found that mesenchymal stem cells and decellularized ECM scaffold had a synergistic effect on promoting skeletal muscle regeneration. The kind of ECM scaffolds features the ability to modulate macrophage phenotype. However, Dearth et al. [52] have shown that COX1/2 inhibitors such as nonsteroidal anti-inflammatory drugs (NSAIDs), frequently seen in clinical practice and common medications like aspirin, were likely to reduce both collagen content and myogenesis in the defect area, which gives an instruction to pay attention when we apply this technique to patients taking these medications in the future. In the last decade, emerging novel graphene oxide scaffolds have been fabricated to stimulate differentiation and proangiogenic activities of myogenic progenitor cells through mechanical interaction with cells [53]. Besides, Zhao et al. proved that dual bioactive dopamine-incorporated electroactive shape memory elastomers could be applied to soft tissue engineering, especially to skeletal muscle regeneration. There are many other instances of application such as flexible electroactive shape memory copolymers, electroactive ductile polylactide copolymers, and injectable self-healing conductive hydrogels [54]. Accordingly, it can be concluded that synthetic composite materials have displayed unique strengths compared with scaffolds with single structures or materials. When selecting scaffold material, we may make a comprehensive consideration and put the advantages of different materials together as possible as we can to create a composite scaffold in order to better promote cell differentiation in muscle. It is also important to make use of the most appropriate scaffold according to the target tissue. The examples mentioned above are summarized in Table 1.

2.5. Environmental Factors Affecting Muscle Differentiation. Muscle stem cells, termed satellite cells, affected by numerous factors, are crucial for skeletal muscle growth and regeneration. The regeneration of skeletal muscle depends on the myogenic differentiation of satellite cells. The most common active promoter of satellite cell proliferation and differentiation in vivo is exercise. One of the most obvious results of exercise is to get function and health state of skeletal muscles improved [55]. The process of myogenic differentiation of stem cells can be divided into two stages. The first stage is cell division, and the second stage is cell differentiation characterized by the expression of certain combinations of myogenic factors [56]. The study of myogenic differentiation of satellite cells has great clinical application potential. For example, this technology may be used to treat VML [9]. When skeletal muscle growth and regeneration are needed, satellite cells will be activated to start myogenic differentiation and then start to proliferate and differentiate into muscle fibers, thus, forming muscle tissue [57]. Pax7 is the guarantee of the function of satellite cells [19]. The growth state of stem cells is closely related to environmental temperature, osmotic pressure, pH value, light, and other factors [58]. For differentiation, the primary importance among them is the mechanical factor because of its role in the cell microenvironment [56, 59].

Table 1: Classification of different scaffolds.

Material of scaffold/scaffold	Feature	Promoting target	Reference
Graphene oxide scaffolds	Exocrine vascular endothelial growth factor (VEGF) secretion	Myogenic progenitor cells	[53]
Hydrogels based on dextran-graft-tetraaniline and N-carboxyethyl chitosan	Degradable conductive and self-healing	C2C12	[54]
Hydrogel core-shell scaffolds combining with nanofiber yarns core	Mimicking native skeletal muscle tissue	C2C12	[45]
Collagen scaffolds with concave microgrooves	Mimicking muscle basement membrane	Myoblasts	[44]
Uniaxially aligned nanofibrous cylinders	Anisotropy and high surface-to-volume ratio	From myoblasts to myotubes	[46]
Nanofiber scaffolds with electrical conductivity property	Presentation of synergistic effects of different materials	Myoblasts	[47]
Porous collagen-GAG scaffolds	Scaffold implantation	VML injury treatment	[49]
Mesenchymal stem cells and extracellular matrix scaffolds	Functioning via promoting macrophage polarization toward the M2 phenotype and suppress macrophage polarization toward the M1 phenotype	Macrophage	[51]

Moreover, the differentiation of satellite cells is able to be regulated or stimulated by sex hormone [60, 61]. Park et al. found that the differentiation of satellite cells can be activated by electrical stimulation [62]. Common metabolites such as lactic acid, polyamine, and metformin can regulate and stimulate myogenic differentiation [63–66]. In addition, r3h domain containing like (r3hdml) and extractive cells, which are closely related to the cells themselves, can also induce the differentiation of stem cells [67, 68].

2.6. Stem Cells for Skeletal Muscle Tissue Engineering

2.6.1. Satellite Cells. Satellite cells, which are also termed muscle stem cells, are located between the basal lamina and sarcolemma of myofibers [69]. The main function of satellite cells is to be responsible for the growth, maintenance, and repair of skeletal muscle after birth, with the ability of self-renewal and differentiation [70]. The paired box transcription factor Pax7 is the specifical gene expressed in satellite cells and is the most important transcription factor to induce satellite cell myogenic differentiation. It is essential for Pax7 to regulate satellite cell expansion and differentiation in both adult and newborn [19, 71]. Pax7 is also absolutely required for skeletal muscle regeneration after acute skeletal muscle injury [72]. H3K4 methyltransferases MLL1 is critical for Pax7 expression and function in vivo. In the absence of MLL1, H3K4me3 at Pax7 and Myf5 promoters are reduced, leading to the decreased expression of Pax7 and Myf5 [18]. It is reported that CD146+ interstitial progenitor cells with no expression of Pax7 have myogenic potential both in vivo and in vitro [57]. MyoD and myf5 are basic regulators determining skeletal muscle lineage in the embryo. They are expressed after muscle injury in satellite cells. The two regulators are essential for muscle regeneration by their stabilizing myogenic identity and giving the capacity for muscle regeneration [73]. CD82 is a novel surface marker for identifying satellite cells isolated from human skeletal muscle. CD82 ensures the expansion and preservation of satellite cells by inhibiting excessive differentiation and it is necessary for satellite cell activation [74–76]. As the adult stem cells of skeletal muscle, satellite cells have been extensively studied and made rapid progress. Prostaglandin E2 (PGE2), which is known as an inflammatory cytokine, can lead to satellite cell expansion by directly targeting satellite cells via the EP4 receptor. Intramuscular delivery of PGE2 can significantly enhance and accelerate the skeletal muscle repair [77]. Notch target genes Hesr1 (Hey1) and Hesr3 (Heyl) are responsible for generating quiescent satellite cells and maintaining the satellite cell numbers [78]. Lysine-specific demethylase 1(Lsd1) can directly regulate key myogenic transcription factor gene to promote muscle regeneration and prevent proadipogenic transcription factor Glis1 differentiating into brown adipocytes [79]. However, although many factors that promote the activation of satellite cells have been researched, they will gradually lose their self-renewal ability as their differentiation. Simultaneously, the main source of satellite cells is skeletal muscle biopsy, and this method will cause great pain to the patient. If a larger amount of satellite cells is needed, it is necessary to biopsy a large number of skeletal muscles, which is almost difficult to achieve clinically. At the same time, the number of satellite cells obtained by the traditional enzymatic dissociation method is small and the purity is low [9]. To solve these limitations, Garcia et al. developed a series of methods for high-grade purification, preservation, and serial transplantation of human satellite cells; these methods provide an accessible system for human satellite cells research and clinical application [80].

2.6.2. Mesenchymal Stem Cells. According to the clarification of The International Society for Cellular Therapy (ISCT), mesenchymal stem cells (MSCs) refer to plastic adherent cells with multidirectional differentiation potential isolated from bone marrow, fat, and other tissues such as umbilical cord blood [81, 82], infrapatellar fat pad [83, 84], and dental

Table 2: Stem cells in myogenic differentiation.

Stem cell types	Stem cell sources	Markers	Advantages
Satellite cells	Muscle biopsy	HEYL, KLF4, MYOD, PAX7, Myf5, CD82	Direct precursor of skeletal muscle
Mesenchymal stem cells	Bone marrow biopsy (BMSC), adipose tissue (ADSC), and other mesenchymal tissues	CD73, CD90, CD105 and lacking CD11b, CD14, CD19, CD34, CD45, CD79a	Easy to obtain and low carcinogenic risk
Induced pluripotent stem cells	Almost every adult tissue	KLF4, c-MYC, OCT4, SOX2	Pluripotent differentiation potential and high differentiation efficiency

tissues [85]. It expresses CD73, CD90, and CD105, but lacking the expression of hematopoietic and endothelial markers CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR. MSC can differentiate into adipocytes, chondrocytes, and osteoblast cell lines in vitro [86, 87]. The two MSCs most commonly used in research are adipose-derived mesenchymal stem cells (ADSCs) and bone marrow-derived stem cells (BMSCs). The bone marrow-derived stem cells are taken from the femur and tibia bone marrow biopsy, which can only obtain a small amount of BMSCs and cause great harm to patients. On the contrary, ADSCs are easier to obtain, faster to grow, and express higher rates of stem cell markers [88]. Thus, the current research about MSCs mainly focuses on ADSCs. Although many studies have shown that MSCs have the effect of promoting muscle regeneration, their mechanism is still unclear. MSCs are multipotent stem cells and have the ability to secrete cytokines and growth factors and have immunoregulatory and proangiogenic abilities [89]. At the same time, it can directly differentiate into muscle tissue in vitro [90]. Under these circumstances, whether MSCs directly differentiate into muscle tissue to replace the damaged muscle tissue or produce paracrine factors to promote muscle regeneration is still controversial. Paracrine factors produced by MSCs such as HGF, bFGF, IGF-1, and VEGF have been confirmed to play key roles in promoting angiogenesis [91]. The latest research found cytokine IL-6 produced by MSCs can stimulate the M2 macrophages to suppress inflammation and regenerate new blood vessels and enhance myogenic differentiation by activating STAT pathway [89, 92]. Mitchell et al. demonstrated ADSCs promote muscle regeneration by its secretome, which contains extracellular vesicle (EV) as well as soluble proteins. EV fraction has anti-inflammatory effects while soluble proteins can reduce the number of senescent cells. Thus, the secretome of ADSCs can promote muscle regeneration both in vivo and in vitro [93]. As for the direct differentiation of MSC into skeletal muscle cells, the current efficiency is still very low. Only 15% of ADSCs can differentiate into skeletal muscle in differentiation medium [94]. Though a number of studies are devoted to promoting its differentiation efficiency, such as culturing cells on scaffolds [95], physical stimulation [96], and chemical stimulation [97]. But the improvement is very limited and not enough to be applied to the clinic. If a paracrine factor that directly promotes differentiation and a method to improve differentiation efficiency can be found, combining the two will greatly promote the application of ADSC in muscle regeneration.

2.6.3. Induced Pluripotent Stem Cells. Induced pluripotent stem cells (iPSCs) were first induced from mouse embryos by introducing specific factors under ES cell culture conditions in 2006 and then induced from adult human fibroblast the next year. Its morphology and growth characteristics are similar to embryonic stem (ES) cells and express ES cell marker genes [98]. Takahashi et al. identified four basic transcription factors, called Yamanaka factors, which must be transformed into starter cells using viral vectors to reprogram the cells into iPSCs: KLF4, c-MYC, OCT4, and SOX2 [98] (see Table 2). Unlike ES cells, iPSCs can derive from almost every adult tissue, and this makes them free of ethical concerns [99]. There are many methods, including transgenic and nontransgenic, to generate a large number of muscle cells from iPSC. Transgenic methods are reliable and can get myogenic progenitors directly. Darabi et al. introduced Pax7 into human ES and iPSC and found that it not only produces a large number of induced myogenic progenitors (iMPCs) with regenerative ability but also contributes to the satellite cell pool and maintains it for a long time after implantation in animals [99]. Culturing iMPC in a 2D environment, it will differentiate into multinucleated myotubes while generating functional skeletal muscle tissues (iSKM bundles) in a 3D hydrogel environment. And iSKM bundles have the biological properties of skeletal muscle such as generating twitch and tetanic contraction. Compared with monolayers in 2D cell culture, iSKM bundles are more similar to native mature muscle. Then, they implanted iSKM bundles into the hindlimb muscle of live mice. Though iSKM bundles are avascular at the first time, ingrown vasculature helped implanted iSKM bundles survival and supported its' function. The 3D culture of IMPCs may be the foundation of PSC-based therapies for muscle regeneration [100]. Nontransgenic methods are easy to do and can be used for research. Shelton et al. developed a protocol for skeletal muscle lineage differentiation from iPSC by using chemically defined media [101]. Wal et al. found that iPSC-derived fluorescence-activated cell sorting-purified myogenic progenitors can expand on a large scale and can develop into striated and contractile myofibers in vitro [102]. To maximum the capacity of unlimited self-renewal and differentiation into any lineage of iPSCs, myogenic progenitors should be produced as pure and easily expandable as possible. CD54, integrin $\alpha 9\beta 1$, and Syndecan2 (SDC2) are the surface markers of Pax7-induced myogenic progenitors. These markers provide a reliable method to purify iPSC-derived myogenic progenitors for real application [100, 103]. Although many studies have confirmed that

iPSC can differentiate into skeletal muscle cells, its disadvantages are also obvious. Immune rejection may be one of the main problems in the clinical application of iPSCs. And due to the inability to precisely control its differentiation direction, iPSC should be thoroughly verified to ensure that they are not carcinogenic [104]. Interestingly, iPSC-derived teratomas show the ability to produce myogenic progenitors. And myogenic progenitors from teratomas can contribute quiescent PAX7+ satellite cells and have functional regenerative capacity [105].

3. Conclusions

Skeletal muscle defects and loss of its function due to various causes including congenital defects, injuries, tumors, degenerative pathologies, and metabolic diseases are really common in the clinic. Besides, the risk of certain muscle diseases increases progressively with age. For example, sarcopenia, a progressive and generalised skeletal muscle disorder involving the accelerated loss of muscle mass and function, is common among adults of older age but can also occur earlier in life. The muscle disease burden arises because of their high prevalence all over the world and close relations to short-term and long-term adverse effects. Although skeletal muscle has the ability of regeneration, it depends on the function of satellite cells. After repeated regenerations, the regeneration ability of satellite cells will gradually be impaired. To make muscle regeneration suitable for clinical use, large-scale expansion of satellite cells or differentiation into myogenic lineage from easily obtained stem cells is the main method for skeletal muscle regeneration. Cells differentiated from stem cells cannot become muscle fibers directly. It is the 3D culture environment that makes it possible for muscle cell transforms into skeletal muscle tissue. But there still remains some limitations for application. On the one hand, although many novel methods can produce a lot more cells than before, the differentiation efficiency is still too low. On the other hand, there is too much reliance on transgenic technology and may cause people to worry about safety.

Conflicts of Interest

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

Authors' Contributions

Dengjie Yu and Zijun Cai contributed equally to this work.

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Research Article

Comparative Analysis of Tenogenic Gene Expression in Tenocyte-Derived Induced Pluripotent Stem Cells and Bone Marrow-Derived Mesenchymal Stem Cells in Response to Biochemical and Biomechanical Stimuli

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The tendon is highly prone to injury, overuse, or age-related degeneration in both humans and horses. Natural healing of injured tendon is poor, and cell-based therapeutic treatment is still a significant clinical challenge. In this study, we extensively investigated the expression of tenogenic genes in equine bone marrow mesenchymal stem cells (BMSCs) and tenocyte-derived induced pluripotent stem cells (teno-iPSCs) stimulated by growth factors (TGF-β3 and BMP12) combined with ectopic expression of tenogenic transcription factor MKX or cyclic uniaxial mechanical stretch. Western blotting revealed that TGF-β3 and BMP12 increased the expression of transcription factors SCX and MKX in both cells, but the tenocyte marker tenomodulin (TNMD) was detected only in BMSCs and upregulated by either inducer. On the other hand, quantitative real-time PCR showed that TGF-β3 increased the expression of *EGR1*, *COL1A2*, *FMOD*, and *TNC* in BMSCs and *SCX*, *COL1A2*, *DCN*, *FMOD*, and *TNC* in teno-iPSCs. BMP12 treatment elevated *SCX*, *MKX*, *DCN*, *FMOD*, and *TNC* in teno-iPSCs. Overexpression of MKX increased *SCX*, *DCN*, *FMOD*, and *TNC* in BMSCs and *EGR1*, *COL1A2*, *DCN*, *FMOD*, and *TNC* in teno-iPSCs; TGF-β3 further enhanced *TNC* in BMSCs. Moreover, mechanical stretch increased *SCX*, *EGR1*, *DCN*, *ELN*, and *TNC* in BMSCs and *SCX*, *MKX*, *EGR1*, *COL1A2*, *DCN*, *FMOD*, and *TNC* in teno-iPSCs; TGF-β3 tended to further elevate *SCX*, *ELN*, and *TNC* in BMSCs and *SCX*, *MKX*, *COL1A2*, *DCN*, and *TNC* in teno-iPSCs, while BMP12 further uptrended the expression of *SCX* and *DCN* in BMSCs and *DCN* in teno-iPSCs. Additionally, the aforementioned tenogenic inducers also affected the expression of signaling regulators *SMAD7*, *ETV4*, and *SIRT1* in BMSCs and teno-iPSCs. Taken together, our data demonstrate that, in respect to the tenocyte-lineage-specific gene expression, BMSCs and teno-iPSCs respond differently to the tenogenic stimuli, which may affect the outcome of their application in tendon repair or regeneration.

1. Introduction

The tendon is a hypovascular tissue transmitting force from the muscle to the bone. It is subject to high risk of injury from acute trauma, overuse, or age-related degeneration. The limited natural healing capacity and the poor functional outcomes of tendon repair are pushing the search for more effective regenerative approaches [1]. Stem cells, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and mesenchymal stem cells (MSCs), possess tenogenic differentiation capacity and have been proposed for tendon repair and regeneration [2]. For example, tendon stem/progenitor cells (TSPCs) showed high capacity to form a tendon-like tissue *in vitro* and *in vivo* [3, 4] and were suggested to be a better cell source for the treatment of tendon disorders than other types of stem cells [5]. However, their application is limited due to the relatively low number within the whole tendon cell population and the loss of phenotype following *in vitro* expansion [6]. Bone marrow-derived MSCs (BMSCs) have been extensively studied for tendon repair in

humans and horses, but the direct use of undifferentiated BMSCs for clinical practice is still debatable partly because of the formation of ectopic bone- or cartilage-like structure at the target sites [7]. iPSCs showed great promise as an emerging cell source for tendon repair [8–10], however, the potential of oncogenic formation is always a concern and more extensive studies are needed before their clinical translation [11]. One alternative way to improve the stem cell-based tendon therapies will be, prior to stem cell implantation, predirecting stem cells toward the tenogenic lineage *in vitro* by using biological (including transcription factors, growth factors, and microenvironment) and biomechanical cues.

Transforming growth factor beta (TGF-β) superfamily of cytokines, including TGF-β subfamily (TGF-β1, TGF-β2, and TGF-β3), bone morphogenic proteins/growth differentiation factors (BMP/GDFs), and activin/inhibin, plays crucial roles in tendon development, homeostasis, and pathogenesis [12–15]. In TGF-β2- and/or TGF-β3-deficient mouse embryos, loss of tendons and ligaments was observed throughout the body, along with no detectable signals of tenocyte-related genes *SCX*, *TNMD*, and *COL1A1* [13]. Targeted deletion of the TGF-β type 2 receptor (Tgfβr2) in tenocytes did not disrupt the tendon differentiation function and growth during embryonic development but destroyed the differentiation markers *SCX*, *TNMD*, and *COL1A1* shortly after birth and reverted the mutant cells to a more progenitor-like state [15]. Moreover, *in vitro* studies revealed that TGF-β2 was able to induce *SCX* expression in embryonic fibroblast cells, mesenchymal stem cell line C3H10T1/2, and mouse limb bud in an organ culture [13]. TGF-β3 was reported to promote tenogenic gene expression in different types of stem cells [14, 16, 17] but its use in iPSCs is very limited. Only one laboratory reported that, with TGF-β3 stimulation, equine iPSCs had a reduced tendon differentiation capacity compared to ESCs [18].

Although BMP/GDFs were originally named for their ability to induce bone formation, the family members BMP12 (GDF7), BMP13 (GDF6), and BMP14 (GDF5) were shown to play important roles in tendon/ligament maintenance and repair [12]. Compared to those in wildtype littermates, the tendons in BMP14$^{-/-}$ and BMP13$^{-/-}$ mice showed similar defects on collagen production and mechanical properties [19, 20]. In BMP12-deficient mice, while the expression of fibrillar collagens and tendon proteoglycans was not affected, the Achilles tendon exhibited a shift towards smaller diameter fibrils that resulted in a small but significant reduction in mean fibril diameter [21]. In *in vitro* studies, although BMP12 has been shown to induce the expression of *TNMD* and *DCN* in equine BMSCs [22] and amniotic fluid-derived MSCs [23], the expression of *SCX* and *TNMD* in canine adipose-derived stromal cells (ASCs) [24], and the expression of *SCX* and *MKX* in human ASCs [25], its application in iPSCs has not been reported yet.

Biophysical force and at least three transcription factors (SCX, MKX, and *EGR1*) are known to be essential for normal tendon development. Previous works from our laboratory and others have demonstrated that mechanical loading and/or ectopic expression of those transcription factors are able to induce the expression of some tenocyte-related genes in MSCs and iPSCs [26–29]. However, it is still largely unknown how a cell behaves under the circumstances of tenogenic stimulation, and the biomarkers specific for tenocyte lineage are also very limited. It is therefore necessary to examine the activities of a great number of tendon-related genes in tenogenic differentiating cells. In the present study, we aimed to compare the *in vitro* tenogenic differentiation capacity of equine tenocyte-derived iPSCs (teno-iPSCs) and BMSCs induced by bioactive molecules TGF-β3 and BMP12 combined with ectopic expression of Mohawk or cyclic uniaxial mechanical stretch. The expression of tenogenic transcription factors (*SCX*, *MKX*, and *EGR1*), tendon extracellular matrix genes (*COL1A2*, decorin (*DCN*), elastin (*ELN*), fibromodulin (*FMOD*), and tenascin C (*TNC*)), and signaling regulators (*SMAD7*, *ETV4*, and Sirtuin1 (*SIRT1*)) was determined. Our goal was to provide valuable information for ongoing and future stem cell-based regenerative treatments of tendon injuries.

2. Materials and Methods

2.1. Cell Culture. Isolation and culture of equine BMSCs were described in our previous study [28]. Briefly, bone marrow aspirates were washed twice with PBS followed by two more washes with basic medium (DMEM/F12 (Invitrogen) with 10% FCS (Gemini) and 1x antibiotics (Gibco)), and then resuspended and cultured in BMSC growth medium (basic medium plus 4 ng/mL bFGF) at 37°C, 5% CO_2. After 72 hours, cells were thoroughly washed with PBS, and fresh medium was added with a change of every 2-3 days. Upon reaching 80-90% confluency, cells (P0) were dissociated with 0.25% trypsin-EDTA and further expanded at a density of 1-2 $\times$ 10^5 cells/cm^2. BMSCs at passages 2-5 were used for experiments. Characterization of mesenchymal stem cell was carried out by flow cytometry with positive expression of CD29, CD44, CD90, CD105, and MHC-I and with negative expression of CD45, CD79, and MHC-II. The multipotency of BMSCs was confirmed by *in vitro* trilineage differentiation using protocols described in our previous work [28] (Supplemental Figure 1).

Generation and multilineage differentiation of teno-iPSCs were also reported in our previous work [28]. Briefly, tenocytes were infected with pHAGE-STEMCCA lentiviruses expressing mouse Oct3/4, SOX2, Klf4, and c-Myc in basic medium for 30 h, and then transferred to mitomycin C inactivated MEF feeder cells in iPSC medium (DMEM containing 10% FCS, 1× NEAA, 1× L-glutamine, 1× sodium pyruvate, 0.055 mM beta-mercaptoethanol, 1000 U/mL of LIF, and 1× antibiotic/antimycotic solution). Medium was replaced every other day. About 10–15 days, individual colonies were manually picked, trypsinized, and further expanded. At passages 3–5, cells were switched to and maintained in feeder-free StemFlex™ medium (Fisher Scientific) and characterized for multilineage differentiation capacity.

2.2. Lentiviral Infection. GFP and equine Mohawk gene were subcloned into replication-defective lentiviral vector pHAGE in which these two genes were separated by IRES (internal

ribosome entry site) element. Lentiviruses expressing GFP (lenti-GFP) alone or MKX and GFP (lenti-MKX) were produced in 293T packaging cells, and supernatant containing the viral particles passed through a Millex-HV 0.45 μm PVDF filter (Millipore, Ireland). Cells seeded on 35 mm culture plates at a density of 20,000 cells/cm^2 the day before infection were exposed to 1 : 1 dilution of filtered viral supernatant in the presence of polybrene (8 μg/mL) for 8 hours, and then cultured in fresh media for 48 hours. The infection efficiency was examined by the expression of GFP signals under fluorescent microscope and qPCR (Supplemental Figure 2). The ectopic expression of Mohawk was determined by qPCR and western blotting.

2.3. Growth Factor Treatment. BMSCs were seeded in 6- or 12-well plates at a density of 1×10^5 cells/cm^2 in BMSC growth medium for two days to reach about 90% confluence. For iPSCs, cells at passages 10-25 were split by 5 mM EDTA and seeded in 6- or 12-well plates at a density of 1×10^5 cells/cm^2 in BMSC basic medium for two days to reach about 90% confluence. Prior to *in vitro* tenogenic differentiation, cells were washed twice with BMSC basic medium, then treated with indicated concentrations of TGF-β3 or BMP12 (PeproTech, Inc., Rocky Hill, NJ) in the same medium for another five days with one medium change two days after the first treatment.

2.4. Sirius Red Staining [30]. After treatment with indicated growth factors, cells grown in 12-well plates were washed twice with PBS, fixed with 70% ethanol for 30 minutes at room temperature, then washed with distilled H2O for three times before incubation with 0.1% Sirius red in saturated aqueous solution of picric acid for one hour. To quantify the stained nodules, the stain was solubilized with 0.2 mL of 0.1% NaOH and absolute methanol (1 : 1 (vol/vol)) for 30 minutes at room temperature. Solubilized stain (0.06 mL) was transferred to wells of a 96-well plate, and absorbance was measured at 540 nm. Data are presented as mean ± SD, $n = 3$.

2.5. Mechanical Stretch. As previously described [28], to test the effects of mechanical force on tenogenic gene expression in teno-iPSCs and BMSCs, cells were seeded on vitronectin-coated poly(ε-caprolactone) (80 kDa; Sigma-Aldrich, St. Louis, MO) nanofibrous scaffolds for 3 days, and then subject to cyclic uniaxial sinusoidal force from a customized bioreactor. The device was programmed to approximate sinusoidal waveforms equating to 3% strain amplitude (0%–6% strain) at a frequency of 1 Hz for 18 hours. At the end of mechanical stretch, samples were lysed in TRIzol reagent for RNA extraction. Static controls were treated identically but with no cyclic mechanical loading.

2.6. RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (qRT-PCR). Samples were lysed in TRIzol reagent (Invitrogen), and total RNA was extracted according to the manufacturer's instruction. One microgram of RNA was then treated with RQ1 RNase-free DNase and then used for cDNA synthesis by using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Equine-specific primer pairs were designed using NCBI primer-blast or published data [18], and the list of primer sequences can be found in Supplemental Table 1. qPCR was carried out using SYBR Green PCR master mix (Biotool, USA) on an Applied Biosystems 7500 real-time PCR system. All PCRs were performed in triplicates. PCR cycle parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. At the end of the program, a melt curve was produced by taking readings every 1°C from 65 to 95°C. The reference gene PSMB2 was used to normalize gene expression, and relative fold changes were calculated using $2^{-\Delta\Delta Ct}$ method.

2.7. Western Blot. Cells were washed twice with PBS and then lysed in ice-cold T-PER buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Milipore Sigma). SDS-PAGE was carried out using the minigel system from Bio-Rad, and proteins were transferred to PVDF membranes. After blocking with TBST containing 5% nonfat dry milk for at least one hour at room temperature, the membranes were incubated at 4°C overnight with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature. After thorough washing with TBST buffer, signals on the membranes were developed with an enhanced chemiluminescent system (Pierce). Antibodies used in this study include the following: scleraxis (Abcepta #AP21316b, 1 : 1000), tenomodulin (Santa Cruz Technology #sc-49325, 1 : 1000), Mohawk (Abcam #ab179597, 1 : 1000), α-tubulin (Cell Signaling Technology #3873, 1 : 1000), p-SMAD3 (Santa Cruz Technology #sc-517575, 1 : 1000), and p-SMAD1/5 (Cell Signaling Technology #9516T, 1 : 1000).

2.8. Statistics. Data were presented as means ± STDEV. Statistical analysis was performed by ANOVA single-factor test in gene expression between the control and treated groups. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Dose Effects of TGF-β3 on Tenogenic Gene Expression in BMSCs and teno-iPSCs. To evaluate the effects of TGF-β3 on tenogenic gene expression, BMSCs and teno-iPSC (clone3, iPSC3) were treated with three different concentrations of TGF-β3 for 5 days, and the expression of tenogenic transcription factors (*SCX*, *MKX*, and *EGR1*), chondrogenic master transcription factor *SOX9*, osteogenic master transcription factor *RUNX2*, and tendon-related ECM genes (*COL1A2*, *DCN*, *ELN*, *FMOD*, and *TNC*) was determined by qPCR. As shown in Figure 1, the levels of *FMOD* and *TNC* in BMSCs and the levels of *SCX*, *FMOD*, and *TNC* in iPSC3 were increased in a dose-dependent manner. The expression of *EGR1*, *SOX9*, and *COL1A2* in BMSCs tended to increase at low concentration of TGF-β3 (4 ng/mL) but were significantly upregulated at higher concentrations of TGF-β3 (20 ng/mL and 100 ng/mL). The expression of *SCX*, *MKX*, *RUNX2*, *DCN*, and *ELN* in BMSCs also trended upwards with the treatment. In iPSC3, TGF-β3 induced significant increase of *EGR1*, *SOX9*, *RUNX2*, *COL1A2*, and *ELN*

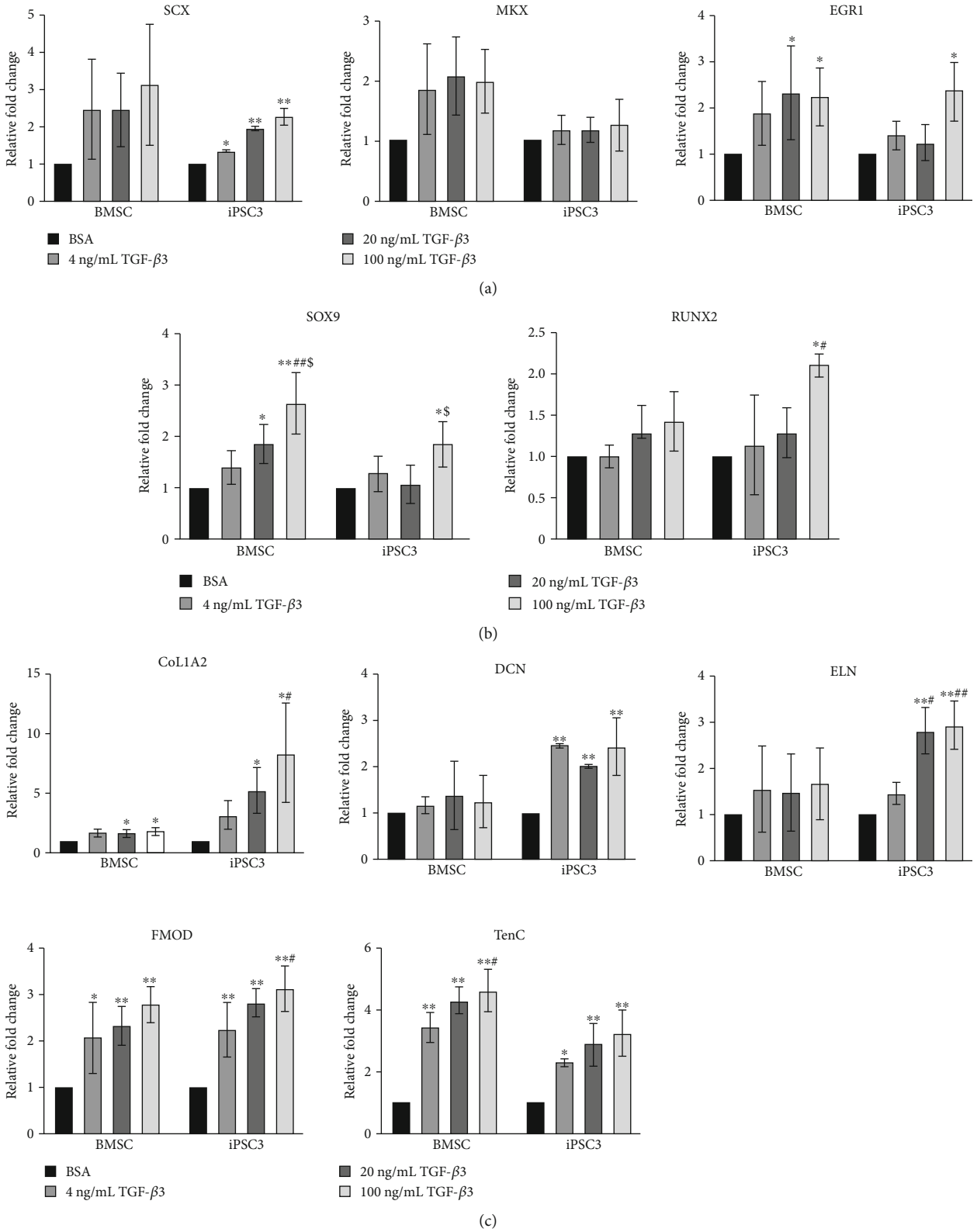

Figure 1: Dose effects of TGF-β3 on tenogenic gene expression in BMSCs and teno-iPSCs. Cells were treated with vehicle medium (0) or various concentrations of TGF-β3 (4, 20, and 100 ng/mL) for 5 days, and cDNA was synthesized from total RNA. Expression of tenogenic transcription factors (a), chondrogenic transcription factor SOX9, osteogenic transcription factor RUNX2 (b) and tenocyte-related ECM genes (c) was determined by qPCR. Relative fold change for each group was calculated by comparison to vehicle medium group, and data for BMSCs were summarized from 3 horses, and data for teno-iPSCs were summarized from 3 passages. *Data were compared to BSA control; #data were compared to the 4 ng/mL group; $data were compared to the 20 ng/mL group.

at higher concentrations (20 ng/mL and/or 100 ng/mL) but not at low concentration. TGF-β3 also dramatically increased the expression of *DCN* at all three tested concentrations, but not in a dose-dependent manner (Figure 1(c)) within this range. Additionally, our previous work has reported that the retention of parental lineage genes varies among teno-iPSC clones and that iPSC3 displays higher levels of tenogenic gene expression than teno-iPSC clone 1 (iPSC1) does [28]. To compare the isogenic differentiation capacity between different iPSC clones, the response of iPSC1 to the tenogenic stimuli was also assessed in this study. As shown in Supplemental Figure 3, iPSC1 showed a similar pattern as iPSC3 on the expression of *SCX*, *SOX9*, and *COL1A2* with TGF-β3 treatment. Moreover, increase of *EGR1*, *RUNX2*, *DCN*, *ELN*, and *TNC* was detected at higher concentrations of TGF-β3. Taken together, these data indicate that the TGF-β3-activated tenocyte-related genes differ from individual cell types.

3.2. Dose Effects of BMP12 on Tenogenic Gene Expression in BMSCs and teno-iPSCs. To assess the effects of BMP12 on the tenogenic differentiation potential of BMSCs and teno-iPSCs, cells were treated with three different concentrations of BMP12 for 5 days, and gene expression was measured by qPCR. As shown in Figure 2, the expression of *SCX*, *MKX*, *EGR1*, *SOX9*, *RUNX2*, *COL1A2*, *DCN*, *ELN*, *FMOD*, and *TNC* tended to increase at all three tested concentrations in BMSCs. On the other hand, BMP12 treatment increased the expression of *DCN* and *TNC* in a dose-dependent manner in iPSC3, where the expression of *SCX*, *MKX*, *COL1A2*, and *ELN* was upregulated by BMP12 at higher concentrations (20 ng/mL and/or 100 ng/mL). As to iPSC1, while the expression of *SCX*, *MKX*, *EGR1*, *DCN*, and *RUNX2* trended upwards, BMP12 significantly increased the expression of *SOX9*, *COL1A2*, and *ELN* at all three concentrations (Supplemental Figure 4). Collectively, these data suggest that, similar to TGF-β3, the BMP12-induced tenocyte-related genes are also varied between cell types.

3.3. Effects of TGF-β3 and BMP12 on Cell Morphology and Tenogenic Protein Expression in BMSCs and teno-iPSCs. As mentioned above, TGF-β3 and BMP12 stimulated the expression of many tenogenesis-related genes at the transcriptional level in BMSCs and teno-iPSCs. The change of intrinsic molecular content may be indicated by alterations in cell morphology. Because the highest concentration (100 ng/mL) greatly increased the expression of *SOX9* and *RUNX2*, growth factors at 20 ng/mL were used for further experiments. Compared to cells treated with BSA vehicle medium, BMSCs and iPSC3 were more inclined to form clusters after treatment with TGF-β3 for 5 days, which was less evident in iPSC1 (Figure 3(a)). Morphology differences were discernible in all the tested cells when they were exposed to BMP12.

To determine the effects of TGF-β3 and BMP12 on the expression of tenogenic proteins in BMSCs and teno-iPSCs, cells were treated with TGF-β3 or BMP12 for 5 days, whole cell lysates were immunoblotted with antibodies against SCX and MKX. As shown in Figure 3(b), both TGF-β3 and BMP12 apparently enhanced the expression of SCX and MKX in BMSCs and two teno-iPSC clones. Tenomodulin is believed to be a marker for mature tenocytes. We failed to measure TNMD gene expression by RT-PCR (data not shown); however, immunoblotting with antibodies against TNMD protein showed specific signals at expected size for cell lysates from BMSCs, and the signals were greatly enhanced by TGF-β3 and BMP12 stimulation. Surprisingly, no TNMD signals were detected in the two teno-iPSC clones with either treatment (Figure 3(b)).

Additionally, to evaluate the effects of TGF-β3 and BMP12 on collagen deposition, treated cells were stained with Sirius red. As shown in Supplemental Figure 5, the intensity of Sirius red staining was significantly increased by TGF-β3 in BMSCs. Quantification data also showed a slight but significant increase of staining in TGF-β3-treated iPSC3 and iPSC1. This effect was not significant with BMP12 treatment in either types of cells.

3.4. Effects of TGF-β3 and BMP12 on Tenogenic Gene Expression in MKX-Overexpressing BMSCs and teno-iPSCs. Our previous work has shown that ectopic expression of Mohawk stimulates the tenogenic gene expression in both BMSCs and teno-iPSCs [28]. In line with this notion, compared to control GFP-expressing cells, overexpression of MKX increased the expression of *SCX*, *EGR1*, *SOX9*, *DCN*, *ELN*, *FMOD*, and *TNC* in BMSCs (MKX-BMSCs) and *COL1A2*, *DCN*, *FMOD*, and *TNC* in iPSC3 (MKX-iPSC3, Figure 4 and Supplemental Figure 6) and iPSC1 (MKX-iPSC1, Supplemental Figure 6 & 7). To determine the synergistic effects of forced expression of MKX with TGF-β3 or BMP12 on tenogenic gene expression, GFP- or MKX-expressing cells were exposed to TGF-β3 or BMP12 for 5 days, and the gene expression was measured by qPCR. As shown in Figure 4, TGF-β3 treatment further enhanced the expression of *EGR1* and *TNC* in MKX-BMSCs and trended to further increase the expression of *SCX*, *SOX9*, *COL1A2*, and *FMOD* in MKX-iPSC3 and *SCX*, *SOX9*, *RUNX2*, *COL1A2*, and *DCN* in MKX-iPSC1. On the other hand, BMP12 treatment trended to increase the expression of *TNC* in MKX-BMSCs, *SCX*, *RUNX2*, *SOX9*, and *COL1A2* in MKX-iPSC3 and *RUNX2*, *COL1A2*, *DCN*, and *TNC* in MKX-iPSC1 (Supplemental Figure 7).

3.5. Effects of Mechanical Stretch on Tenogenic Gene Expression in TGF-β3- and BMP12-Treated BMSCs and teno-iPSCs. Both molecular cues and mechanical loading play essential roles in tendon development and homeostasis. Our previous study has reported that mechanical stretch affects tenogenic gene expression in BMSCs and teno-iPSCs [28]. In accordance with this statement, compared to static condition, cyclic uniaxial stretch increased the expression *SCX*, *EGR1*, *DCN*, *ELN*, and *TNC* in BMSCs and *SCX*, *MKX*, *EGR1*, *SOX9*, *COL1A2*, *DCN*, *FMOD*, and *TNC* in iPSC3. To determine the synergistic effects of mechanical stretch with TGF-β3 or BMP12 on tenogenic gene expression, cells were pretreated with TGF-β3 or BMP12 prior to cyclic uniaxial mechanical tensile, and the expression of tenocyte-related genes was determined by qPCR. As shown

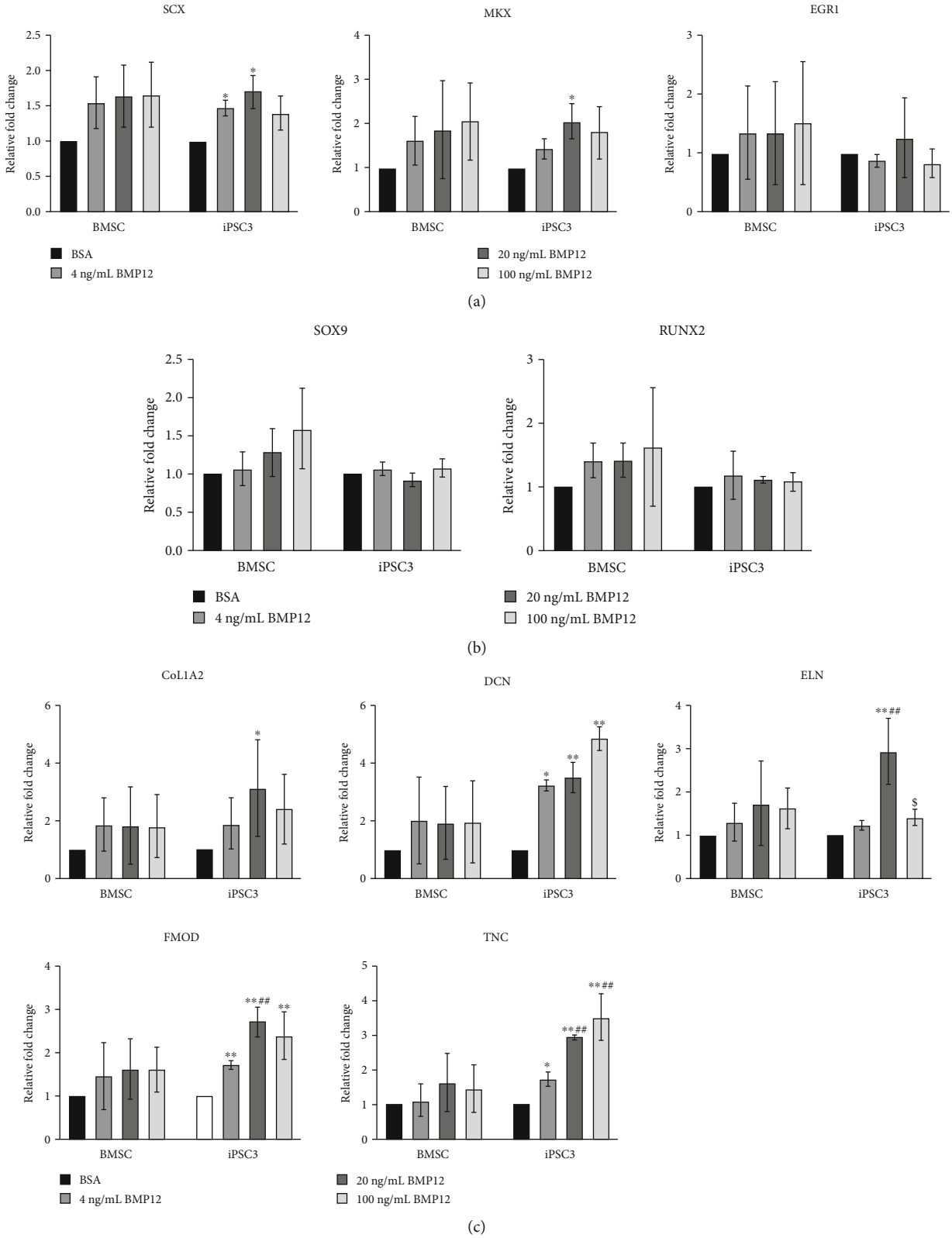

Figure 2: Dose effects of BMP12 on tenogenic gene expression in BMSCs and teno-iPSCs. Cells were treated with vehicle medium (0) or various concentrations of BMP12 (4, 20, and 100 ng/mL) for 5 days, and cDNA was synthesized from total RNA. Expression of tenogenic transcription factors (a), SOX9 and RUNX2 (b), and tenocyte-related ECM genes (c) was determined by qPCR. Relative fold change for each group was calculated by comparison to vehicle medium group. *Data were compared to BSA control; #data were compared to the 4 ng/mL group; $data were compared to the 20 ng/mL group.

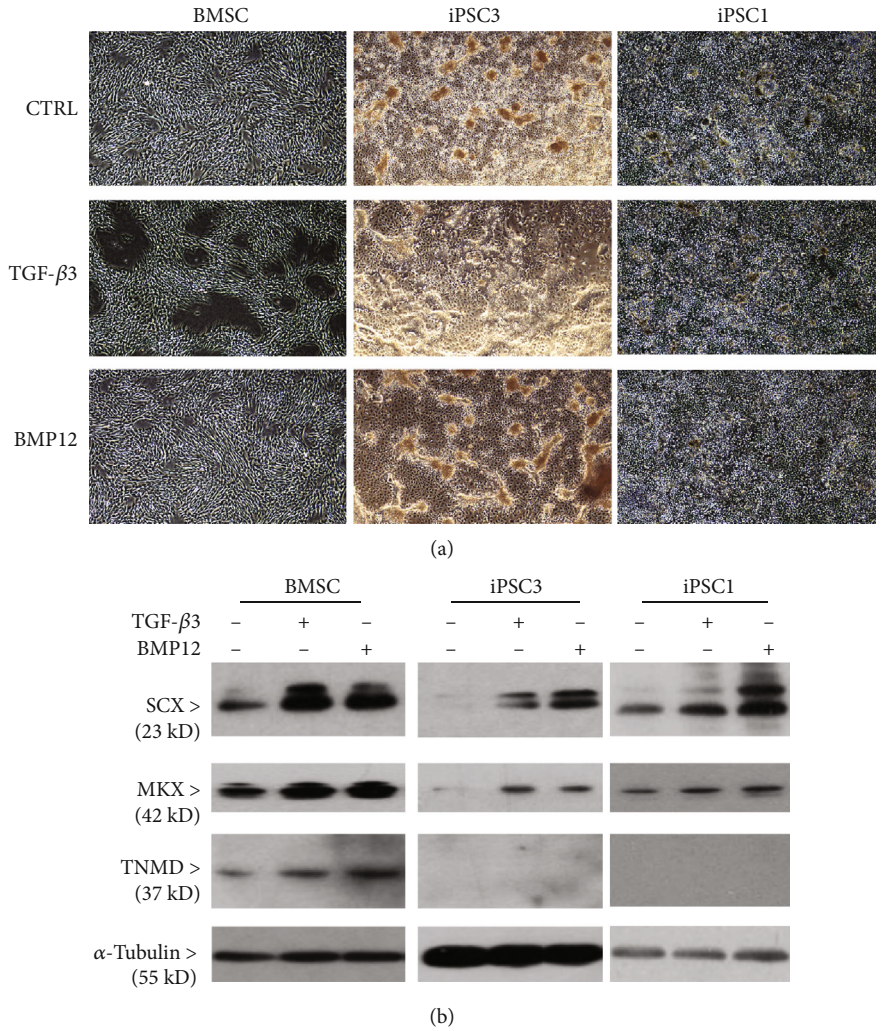

Figure 3: Effects TGF-β3 and BMP12 on cell morphology and tenogenic protein expression. BMSCs and tow teno-iPSC clones (iPSC3 and iPSC1) were treated with vehicle medium, TGF-β3 (20 ng/mL), or BMP12 (20 ng/mL) for 5 days. The cell morphology was imaged (a), and whole cell lysates were blotted for scleraxis (SCX), Mohawk (MKX), tenomodulin (TNMD), and α-tubulin (b).

in Figure 5, TGF-β3 increased the expression of *SCX*, *MKX*, *EGR1*, *SOX9*, *COL1A2*, *FMOD*, *ELN*, and *TNC* in static BMSCs and *SCX*, *COL1A2*, *DCN*, and *TNC* in static iPSC3. Exposure of TGF-β3 pretreated cells to mechanical stretch increased the expression of *SCX*, *MKX*, *SOX9*, *COL1A2*, *ELN*, and *TNC* in BMSCs and *SCX*, *MKX*, *RUNX2*, *COL1A2*, *DCN*, and *TNC* in iPSC3. On the other hand, BMP12 treatment elevated the expression of *SCX*, *COL1A2*, *ELN*, and *FMOD* in static BMSCs and *SCX* and *COL1A2* in static iPSC3. Mechanical loading on BMP12-pretreated cells upregulated the levels of *EGR1*, *DCN*, and *TNC* in BMSCs and *EGR1* and *DCN* in iPSC3. Taken together, these data indicate that mechanical stretch and growth factors synergistically regulate tenogenic gene expression in a cell type-dependent manner.

3.6. Potential Signaling Networks Associated with Tenogenic Gene Expression in BMSCs and teno-iPSCs. TGF-β ligands phosphorylate and activate the receptor-regulated transcription factors SMAD2/3 or SMAD1/5/8 via binding to transmembrane TGF-β receptors [31]. As expected, in all tested cells TGF-β3 and BMP12 greatly enhanced the phosphorylated form of SMAD3, and SMAD1/5, respectively (Figures 6(a) and 6(b)). It has also been acknowledged that the TGF-β superfamily regulates cell proliferation and differentiation through not only the canonical SMAD signaling but also the SMAD-independent noncanonical pathways [32]. In line with this notion, the mRNA levels of *SMAD7*, one inhibitory Smad that negatively controls both TGF-β and BMP-induced SMAD signaling [33], were significantly increased by TGF-β3 at higher concentrations in BMSCs and teno-iPSCs (Figure 6(c)). These phenomena were not observed when cells were treated with BMP12 or overexpressing MKX (Figures 6(d) and 6(e)). Interestingly, mechanical stretch resulted in an apparent increase of *SMAD7* expression in BMSCs, but not in iPSCs (Figure 6(f)), suggesting that regulation of TGF-β signaling by mechanical force might be cell type dependent. In addition, the expression of *ETV4*, a gene that can be used as transcriptional readout of ERK/MAPK activity [34], was highly upregulated by TGF-β3 in BMSCs

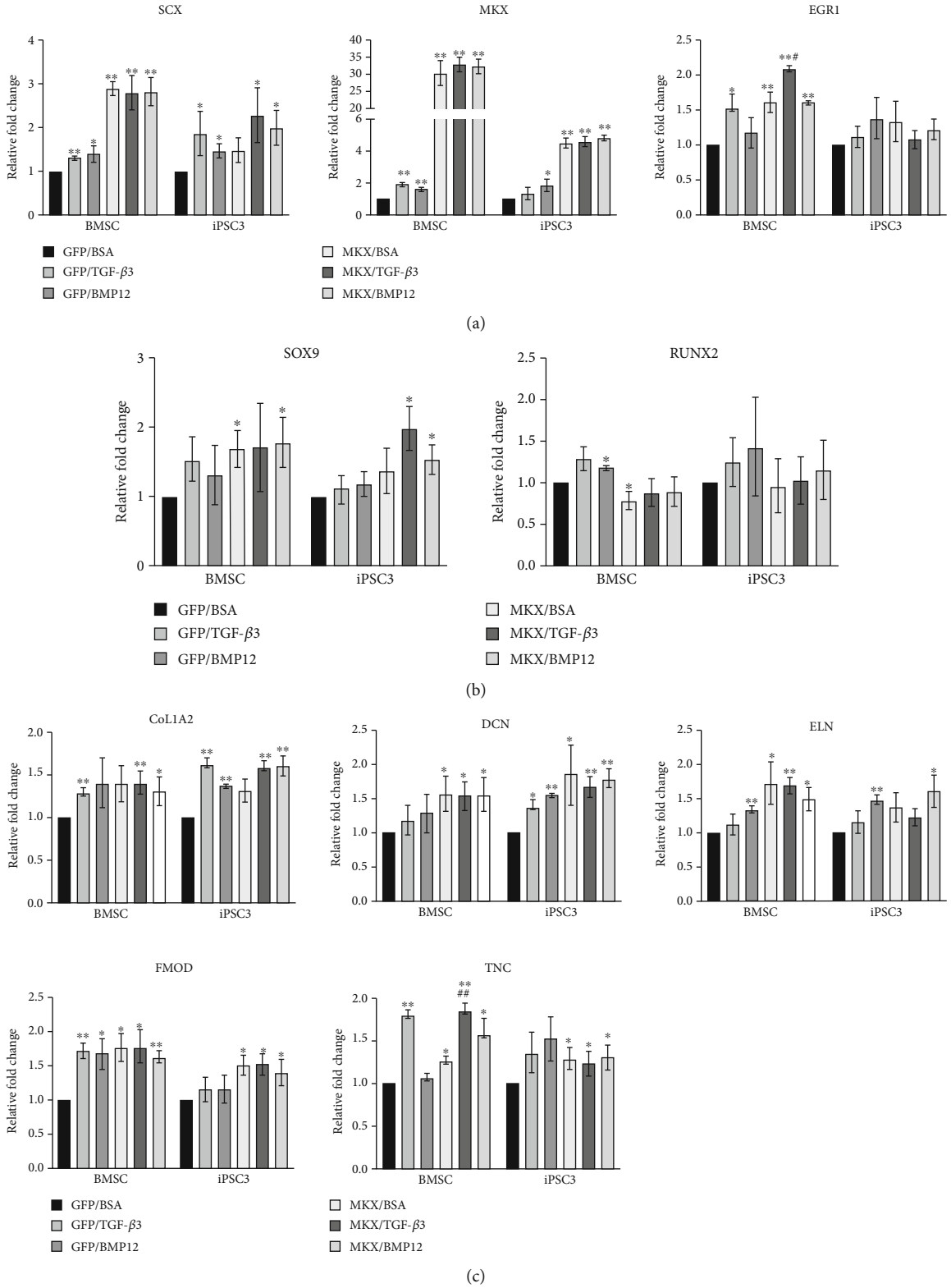

Figure 4: Effects of TGF-β3 or BMP12 on tenogenic gene expression in MKX-overexpressing BMSCs and teno-iPSCs. Cells expressing GFP or equine Mohawk were treated with vehicle medium (GFP/BSA and MKX/BSA), TGF-β3 (20 ng/mL, GFP/TGF-β3 and MKX/TGF-β3), or BMP12 (20 ng/mL, GFP/BMP12 and MKX/BMP12) for 5 days. cDNA was synthesized from total RNA, and expression of tenogenic transcription factors (a), SOX9 and RUNX2 (b), and tenocyte-related ECM genes (c) was determined by qPCR. Relative fold change for each group was calculated by comparison to the GFP-CTRL group. *Data were compared to the GFP/BSA group, and #data were compared to the MKX/BSA group.

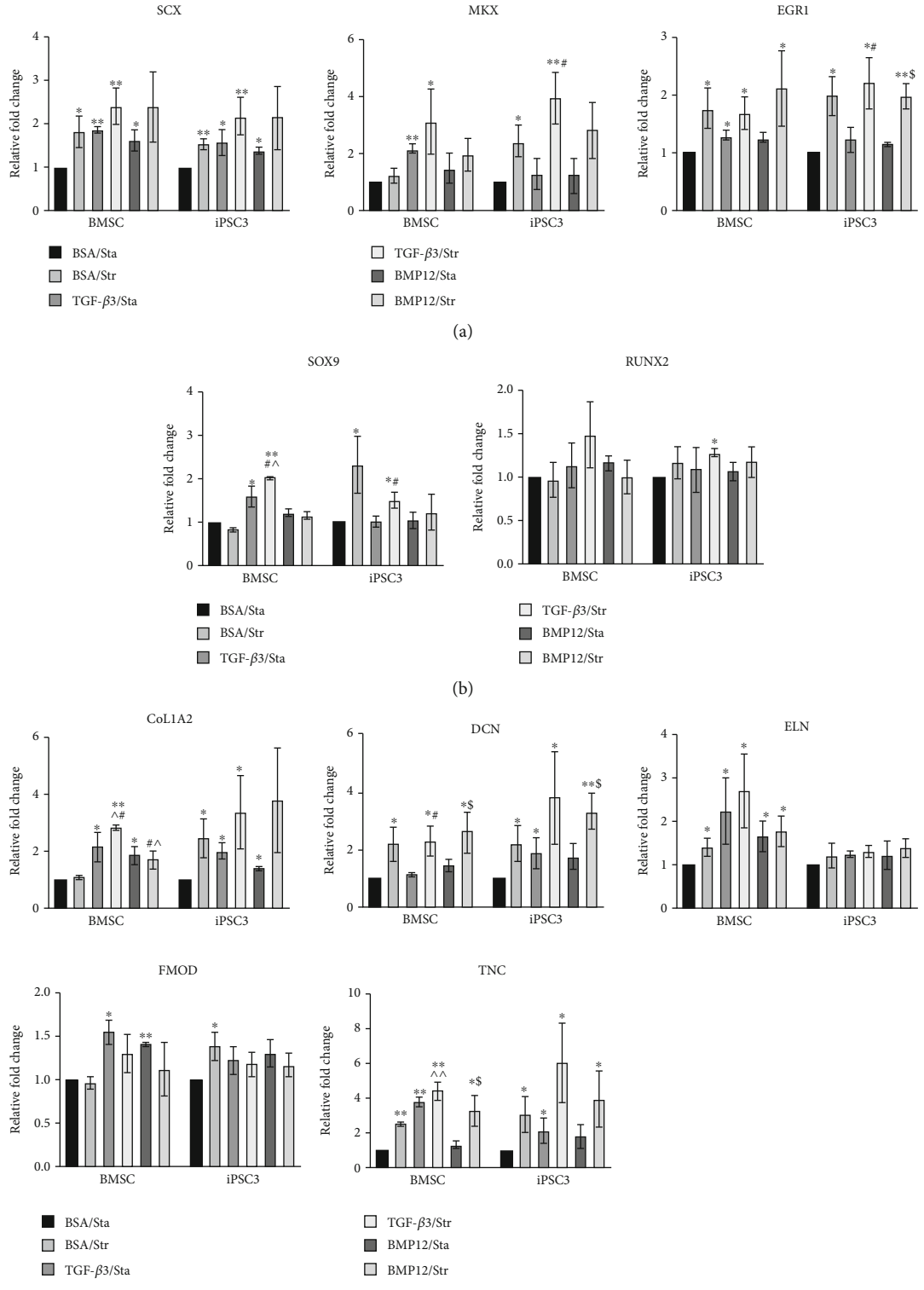

Figure 5: Effects of cyclic uniaxial mechanical stretch on gene expression in TGF-β3- or BMP12- treated BMSCs and teno-iPSCs. BMSCs and teno-iPSCs were seeded on vitronectin-coated PCL scaffolds for 2 days in basic medium, and then treated with vehicle medium, TGF-β3, or BMP12 for 2 days prior to uniaxial mechanical stretch for 18 hours in the presence of vehicle medium (BSA/Str), TGF-β3 (TGF-β3/Str), or BMP12 (BMP12/Str). Cells seeded on PCL scaffolds without mechanical loading but with vehicle medium (BSA/Sta), TGF-β3 (TGF-β3/Sta), or BMP12 (BMP12-Sta) were served as static control. Expression of tenogenic transcription factors (a), SOX9 and RUNX2 (b), and tenocyte-related ECM genes (c) was determined by qPCR, and relative fold change for each group was calculated by comparison to BSA/Sta group. *Data were compared to the BSA control group; ^data were compared to BSA/Str; $^{\$}$data were compared to BMP12/Sta.

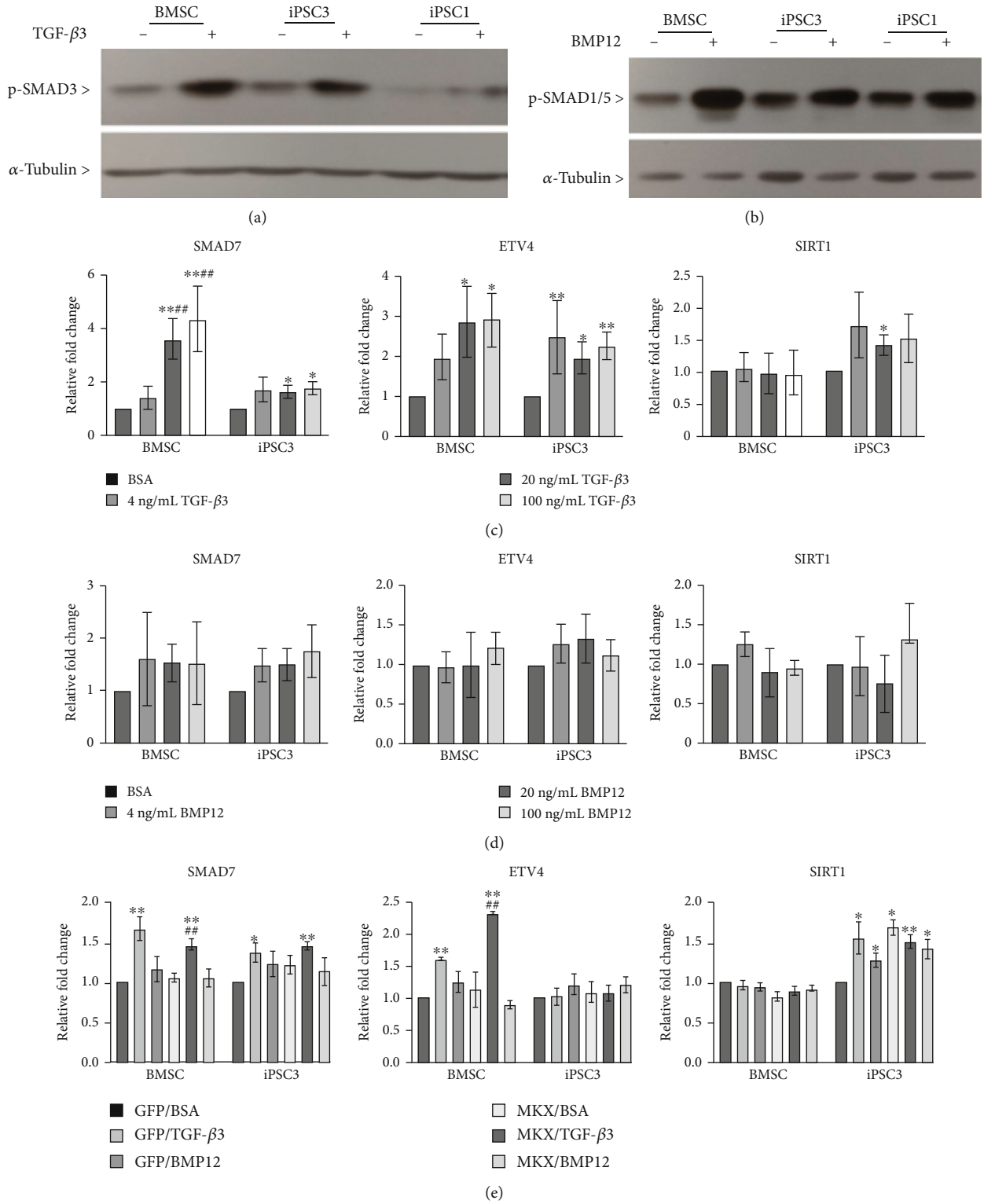

Figure 6: Continued.

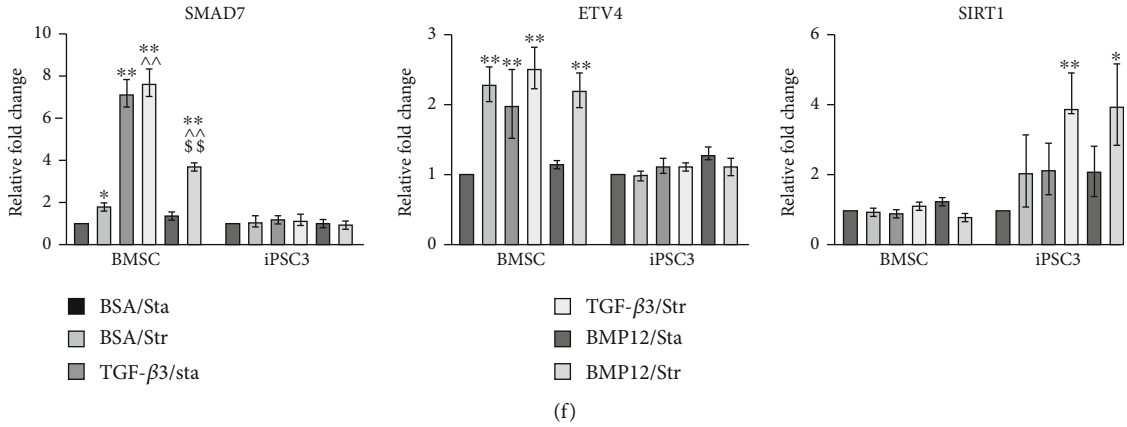

Figure 6: Effects of tenogenic stimuli on the expression of signaling factors. (a) BMSCs and teno-iPSC were treated with vehicle medium or TGF-β3 (20 ng/mL) for 1 hr, and whole cell lysates were blotted for phosphorylated SMAD3 and a-tubulin. (b) BMSCs and teno-iPSCs were treated with vehicle medium or BMP12 (20 ng/mL) for 1 hr, and whole cell lysates were blotted for phosphorylated SMAD3 (p-SMAD3) and a-tubulin. (c) Cells were treated as in Figure 1, and the expression of *SMAD7*, *ETV4*, and *SIRT1* was determined by qPCR. *Data were compared to BSA control; #data were compared to the 4 ng/mL group. (d) Cells were treated as in Figure 2, and the expression of *SMAD7*, *ETV4*, and *SIRT1* was determined by qPCR. (e) Cells were treated as in Figure 4, and the expression of *SMAD7*, *ETV4*, and *SIRT1* was determined by qPCR. *Data were compared to the GFP/BSA group, and #data were compared to the MKX/BSA group. (f) Cells were treated as in Figure 5, and the expression of *SMAD7*, *ETV4*, and *SIRT1* was determined by qPCR. *Data were compared to the BSA control group; ^data were compared to BSA/Str; $data were compared to BMP12/Sta.

and iPSC3 but not in iPSC1 (Figure 6(c), Supplemental Figure 1). Meanwhile, activation of *ETV4* was also revealed in BMSCs by mechanical force (Figure 6(f)), but not in cells treated by either BMP12 or ectopic expression of MKX (Figures 6(d) and 6(e), Supplemental Figures 2 and 4), implying that activation of ERK/MAPK signaling is dependent on cell type as well as on tenogenic inducers. On the other hand, to understand whether an epigenetic modifier was affected by tenogenic inducers, the transcriptional activities of sirtuin-1 (*SIRT1*), one of the NAD-dependent histone deacetylases (HDACs), were determined by qPCR on stimulated cells. The results showed that *SIRT1* expression in BMSCs and iPSC1 was barely affected by any of the tested stimuli. However, its level in iPSC3 was slightly but significantly elevated by TGF-β3 at 20 ng/mL, overexpression of MKX alone or combined with TGF-β3 or BMP12, and mechanical stretch combined with TGF-β3 or BMP12 (Figure 6 and Supplemental Figure 4), suggesting that activation of *SIRT1* gene by tenogenic stimuli is dependent on intracellular context.

4. Discussion

In this study, we extensively examined the effects of individual or combined tenogenic cues, including TGF-β3, BMP12, ectopic expression of MKX, and mechanical stretch, on the expression of tenocyte-related genes in teno-iPSCs and BMSCs. Our data revealed that those stimuli affected the activities of tenogenic transcription factors, including *SCX*, *MKX*, and *EGR1*, and the expression of tendon-related ECM genes, such as *COL1A2*, *DCN*, *ELN*, *FMOD*, and *TNC*. Moreover, those tenogenic inducers also showed high impact on the expression of signaling regulators *SMAD7*, *ETV4*, and *SIRT1* in BMSCs and teno-iPSCs.

4.1. Regulation on Tenocyte-Associated Transcription Factors. Although the exact mechanisms triggering tenogenesis still remain elusive, to date, at least three transcription factors, i.e., SCX, MKX, and *EGR1*, have been reported to play essential roles in tendon development. Depletion of either genes caused apparent tendon abnormalities [35–38]. In other words, stimulation of those genes may drive the stem cell fate to tenocyte lineage. As the first transcription factor found to be required for tendon formation, SCX is a widely accepted tenogenic marker in *in vitro* studies. While it is still not fully understood how *SCX* activity is regulated *in vivo*, the loss of SCX signals in TGF-$\beta 2^{-/-}$- and TGF-$\beta 3^{-/-}$-deficient mouse embryos suggest that TGF-β signaling is needed for *SCX* expression in developing tendon [13]. In our study, treatment with TGF-β3 or BMP12 greatly increased *SCX* expression at the protein level in both teno-iPSCs and BMSCs, suggesting TGF-β ligands may be served as a potent tenogenic inducer to program stem cells towards tenocytes. Moreover, our study also showed that cyclic mechanical loading alone (1.0 Hz with 0%-6% sinusoidal wave of strain for 18 hrs) was able to enhance the expression of *SCX* in both BMSCs and teno-iPSCs. This is in line with the notion that mechanical stress is an inducer of *SCX* expression [39], although another study from Brown et al. reported that it was not mechanical stress alone (1 hr/day of 0.5 Hz with 1% strain for 3 days), but TGF-β2 or TGF-β2 combined with mechanical stress that increased *SCX* expression in mouse BMSCs [40]. This discrepancy may be due to the different stretch parameters applied. Nevertheless, an earlier study from Maeda et al. showed that physical forces could regulate the release of active TGF-β from ECM, thus fine-tune *SCX* expression through TGF-β/SMAD2/3-mediated signaling [41]. This might also be the reason for the synergistic effects of TGF-β3 and mechanical loading on *SCX* expression in both BMSCs and teno-iPSCs. Additionally, our data also

demonstrated that *SCX* expression could be promoted by forced expression of *MKX*, especially in BMSCs. This is in agreement with the report that ectopic expression of MKX dramatically increased the level of *SCX* in C3H10T1/2 cells through TGF-β signaling [42], but not in accord with other studies where MKX did not activate the expression of *SCX* in human BMSCs [27], or in mouse periodontal ligament (PDL) fibroblasts [43] or Achilles tendons [44]. This disparity suggests that the capability of MKX to regulate *SCX* or other targets may differ between species and cell types.

On the other hand, while MKX is highly expressed in developing tendons and plays important roles in tenogenic differentiation, there is very limited information on its upstream regulator(s) [45]. BMP12 has been reported to be one of the growth factors able to activate *Mkx* in a variety of mesenchymal stem cells [27, 46, 47]. In our study, this effect was not evident with qPCR analysis; however, results from western blotting showed apparent higher levels of MKX in BMP12- or TGF-β3-treated BMSCs and teno-iPSCs than those in vehicle controls, implying a role of TGF-β signaling in the control of *MKX* expression. Furthermore, *MKX* activation can also be induced by mechanical loading as it was greatly elevated in rat patellar tendon-derived cells upon exposure to mechanical tensile (4% mono-axial cyclic elongation for 6 hrs) [37]. An *in vivo* study from Kayama et al. also showed increased level of *MKX* in treadmill mouse Achilles tendon [45]. The authors further reported that mechanical stretch (0.25 Hz with 2% strain for 6 hrs) induced the nuclear translocation of transcription factor Gtf2ird1 in rat primary Achilles tenocytes, thus boosted the expression of *MKX*. In our study, with respect to *MKX* expression, mechanical loading showed more significant effects in teno-iPSCs than that in BMSCs. It will be of great interest to know whether *Gtf2ird* also mediates the biomechanical responses in those cells.

In addition to SCX and MKX, the zinc finger transcription factor *EGR1* also appears to play important roles in controlling tendon development, homeostasis, and repair [29, 38, 48]. It is known that *EGR1* can be induced in various tissues by multiple extracellular stimuli, such as growth factors and mechanical signals. However, it remains unclear how *EGR1* is regulated by biochemical cues during tendon formation and *in vitro* tenogenic differentiation. Guo et al. reported that *EGR1* level was highly enhanced in rat TPSCs treated with 10 ng/mL TGF-β1 for 10 days [5], but the study from Yin et al. showed decreased expression of *EGR1* in rat BMSCs treated with the same concentration of TGF-β1 for 3 or 7 days [30]. Another study from Guerquin et al. showed no changes on *EGR1* expression in C3H10T1/2 cells treated with 20 ng/mL TGF-β2 for 1 or 24 hrs [38]. In our study, enhanced expression of *EGR1* was observed in equine BMSCs treated with TGF-β3 at 20 ng/mL or 100 ng/mL and in teno-iPSCs treated with 100 ng/mL TGF-β3. These data suggest that induction of *EGR1* by TGF-β may be cell type and concentration dependent. Additionally, BMP12 was also reported to be able to induce *EGR1* expression in turkey BMSCs [49]. However, in our study, BMP12 only tended to increase *EGR1* in teno-iPSC1 but not in BMSCs and teno-iPSC3. These results are partially in line with the findings from the other study where *EGR1* expression in rat BMSCs was not influenced by BMP12 [30]. Of note, as *EGR1* is a well-known mechanosensitive gene, it is expected to observe apparent increase of *EGR1* in BMSCs and teno-iPSCs upon mechanical loading, which may override the effects resulted from another stimulus.

It is also worth noting that the tenogenic stimuli used in our study influenced the activities of chondrolineage-related transcription factor *SOX9* and osteolineage-related transcription factor *RUNX2*. For example, the level of *SOX9* in BMSCs was dose dependently upregulated by TGF-β3 and trended upwards by BMP12, while in teno-iPSC3, it was elevated by a high dose of TGF-β3 and/or mechanical stretch. Moreover, the expression of *RUNX2* was decreased in BMSCs and teno-iPSC1 by ectopic expression of MKX, but increased in teno-iPSCs by a high dose of TGF-β3. These results are not surprising because TGF-β signaling, mechanical loading, and MKX are also known to play important roles in regulating cartilage and bone formation [37, 50]. Nevertheless, our results indicate that forced expression of MKX may attenuate the risk of bone formation in tendon repair with certain types of stem cells that are preprogrammed by growth factors.

4.2. Regulation on Tendon-Related Extracellular Matrix Gene Activity. Precisely organized tendon matrix is synthesized by tendon cells and predominantly composed of type I collagen, together with small amount of other types of collagens and noncollagenous materials [51]. Tendon injury is usually associated with disrupted structures, and the repair/healing process is involved in rebuilding the injured tissue back with normal functions. Hence, although many of them are not tendon specific, the expression of ECM-related genes is often used as reference to evaluate the potential of stem cell therapy for tendon disorders. In this study, we determined the levels of ColI subunit *COL1A2*, decorin (*DCN*), elastin (*ELN*), fibromodulin (*FMOD*), and tenascin-c (*TNC*) in stimulated BMSCs and teno-iPSCs. Our data revealed that *COL1A2* was upregulated in all the tested cells by treatment involved with TGF-β3. This is consistent with the fact that TGF-β stimulates the binding of ubiquitous transcription factor Sp1, the SMAD3/4 complex, and the coactivators p300/CBP to *COL1A2* promoter [52]. Moreover, in teno-iPSCs, *COL1A2* was also activated by overexpression of MKX, BMP12/MKX, or mechanical stretch. Since the expression of MKX in teno-iPSCs was enhanced by mechanical loading, it is reasonable to presume that the activity of *ColA2* in teno-iPSCs can be regulated by MKX. Indeed, previous studies have shown that *COL1A2* level was decreased in *MKX*$^{-/-}$ mice [43, 44] and increased in MKX-overexpressing PDL fibroblasts [43]. Our data also demonstrated that overexpression of MKX alone or combined with TGF-β3 or BMP12 increased or trended to increase the expression of *COL1A2*, *DCN*, *ELN*, *FMOD*, and *TNC* in all the tested cells, further supporting that MKX plays crucial roles in regulating ECM gene activities in BMSCs and teno-iPSCs.

Decorin (DCN), the most abundant noncollagenous matrix protein in the tendon [53], participates in collagen fibril organization and prevents fibrosis formation [54]. In the present study, *DCN* level was increased in both BMSCs

and teno-iPSCs by mechanical tensile-related inducer. This is partially in agreement with the study from Youngstrom et al. but contradictory to other studies where mechanical stimulation decreased *DCN* expression in human primary rotator cuff fibroblasts and C3H10T1/2 cell lines [55–57]. Another study from Chen et al. showed no changes on *DCN* level when human ES-derived MSCs were subject to mechanical stress for 24 hrs [26]. These inconsistencies may be due to different stretch parameters applied. Indeed, Xu et al. reported that *DCN* expression was increased by moderate treadmill running but decreased by strong treadmill running in rat Achilles tendon [58]. Of note, our results also revealed that treatment with TGF-β3 or BMP12 resulted in a significant elevation of *DCN* in teno-iPSCs but not in BMSCs, suggesting that regulation of *DCN* activity by TGF-β signaling is cell type dependent.

Fibromodulin is reported to be essential for the maintenance of tendon stem cell niches [3], and its deficiency resulted in a structurally and mechanically abnormal tendon phenotype [59]. Xu et al. reported that cyclic tensile strain induced the expression of *FMOD* in rat TPSCs [60]; however, our data demonstrated that mechanical loading showed little effects on *FMOD* activity in BMSCs and teno-iPSCs, suggesting that regulation of FMOD expression by mechanical force also varies on cell type. In addition, the study from Tan et al. showed that targeted deletion of TGF-βr2 decreased the level of *FMOD* in mouse tenocytes [15], suggesting that TGF-β signaling is involved in *FMOD* activity. Indeed, in our study, its level was upregulated in TGF-β3-treated BMSCs and in TGF-β3- or BMP12-treated teno-iPSCs.

Elastin is the core protein of elastic fibers with unique ability to sustain large deformation [61]. While disrupted elastic fibers are associated with the development of chronic tendinopathy [62], increased expression of elastin in injured tendons suggests that it may play a role in the healing process [38, 63]. In *MKX*$^{-/-}$ mouse Achilles tendon, *ELN* level was much higher than that in the wildtype, indicating MKX may repress *ELN* gene activity [44]. Our data, however, is somewhat contradictory to that finding as overexpression of MKX elevated the level of *ELN* in BMSCs but not in teno-iPSCs, suggesting that the target(s) of transcription factor MKX is cell type dependent. In addition, whilst Min et a.l reported that mechanical strain downregulated the expression of *ELN* in human parametrial ligament fibroblasts [64], our results demonstrated that mechanical tensile-related inducers upregulated *ELN* in BMSCs. This inconsistency implied that the response of *ELN* gene to biophysical force may also rely on the cell type.

Tenascin C is expressed relatively low in mature tendon and suggested to play a role in proper alignment and orientation of collagen fibrils within the tendon [65]. Significant increase of *TNC* in acutely injured equine tendon indicates that it may also contribute to tendon repair [66]. Previous studies have shown that *TNC* activity can be affected by both biochemical and biomechanical cues [67]. In our study, all the tested stimuli enhanced the expression of *TNC* in teno-iPSCs, and similar results were obtained from BMSCs treated with all stimuli except BMP12. Although these data did not agree with the studies showing decreased *TNC* in mechanical stressed C3H10T1/2 cells [57], increased *TNC* in BMP12-treated rat BMSCs [46], and variable expression of *TNC* in TGF-β3-treated equine ESCs and iPSCs [18], they are in agreement with other studies reporting increased *TNC* in mechanical strained human BMSCs [68] and in TGF-β3-treated equine ADMSCs [69].

Tenomodulin, one of the transmembrane glycoproteins, has been widely accepted as a specific marker for tenogenic differentiation because it is predominantly expressed in tendon and ligament. SCX is so far the only transcription factor found to directly transactivate *TNMD* via E-boxes to positively regulate tenocyte differentiation and maturation [70]. In our study, although for unknown reason, qPCR with several sets of primers failed to detect *TNMD* at the RNA level in any type of cells used in this study, results from western blotting revealed apparent increases of SCX and TNMD in TGF-β3- or BMP12- treated BMSCs. On the other hand, Kayama et al. showed that deletion of *MKX* upregulated the expression of *SCX* but not that of *TNMD* in mouse Achilles tendon [45], implying that activation of *SCX* does not always correlate with the expression of *TNMD*. In the current study, in spite of evident expression of SCX, no TNMD was detected in either control or stimulated teno-iPSCs, suggesting a cofactor(s), which is likely not expressed or insufficient in teno-iPSCs, might be required for SCX-mediated *TNMD* activation.

Taken together, our results demonstrated that activation of tenogenic genes was not only dependent on the inducers but also varied between cell types. In respect to the expression of mature tenocyte marker TNMD and to the reduction of osteogenic gene expression, activation of TGF-β signaling by TGF-β3 or BMP12 combined with ectopic expression of transcription factor Mohawk may be suitable for BMSCs towards the tenocyte-lineage differentiation. However, the lack of TNMD expression in teno-iPSCs requires further work to optimize the condition for their tenogenic differentiation.

4.3. Regulation on Potential Signaling Factors. The molecular mechanisms underlying tendon development are generally thought to play similar roles in adult tissue regeneration. Upon injury, a variety of growth factors and cytokines are released from the injured tendons and adjacent tissues [71], and different signaling pathways, including TGF-β-SMAD2/3, BMP-SMAD1/5/8, ERK/MAPK, mTOR, and Wnt/β-catenin, are reported to associate with tendon development and repair [34, 72–74]. *SMAD7* is known to be a TGF-β-inducible antagonist of TGF-β signaling [33]. It can also be induced by other cytokines and growth factors, such as interferon-γ, tumor necrosis factor-α, and epidermal growth factor [75–77], suggesting that *SMAD7* is linked in crosstalk between divergent signaling pathways. In the present study, TGF-β3 stimulated the expression of *SMAD7* in both BMSCs and teno-iPSCs, implying a modulatory role of *SMAD7* in the negative feedback loop. Moreover, since mechanical loading is known to positively regulate TGF-β signaling, it is therefore not surprising to see increased level of *SMAD7* in stretched BMSCs. Interestingly, this effect was not observed in teno-iPSCs. One possible reason is that the

biochemical signals converted from the mechanical force used in this study are inadequate to stimulate/maintain the expression of *SMAD7* in teno-iPSCs.

ETV4 is a member of the ETS domain transcription factor family. Its transactivation capacity is enhanced following activation of the ERK- and JNK- MAPK signaling pathways [78], thus can be served as transcriptional readout of ERK/MAPK activity. In the current study, *ETV4* was upregulated by TGF-β3 in both BMSCs and iPSC3, indicating the intracellular crosstalk between the ERK and TGFβ signaling pathways. Moreover, mechanical stretch has been shown to induce ERK1/2 phosphorylation in primary tendon fibroblasts [79], and our data also revealed an increase of *ETV4* expression in mechanical loaded BMSCs, suggesting that the ERK signaling can be activated by mechanical force. On the other hand, similar to the finding that ERK1/2 was not activated by mechanical force in human dermal keratinocyte cells [80], stretch-induced expression of *ETV4* was not evident in teno-iPSCs, implying that the mechanical loading-mediated ERK activation may be cell type dependent. Further work will be required to understand the roles of ERK signaling pathway in stem cell-based tenogenic differentiation.

Sirtuin-1 (*SIRT1*) is an NAD^+-dependent class III HDAC targeting both histone and nonhistone proteins. It has been shown to inhibit the apoptosis and inflammatory response in human tenocytes [81] and to mediate the activation of immune/defense genes induced by mechanical stretch in human PDL cells [82]. Interestingly, while the class I/II HDAC inhibitors trichostatin A and valproic acid promoted SCX expression in mouse TSPCs [83], overexpression of *SIRT1* also upregulated *SCX* in rat BMSCs where the *SIRT1*-JNK/SMAD1-PPARg signaling pathway was accounted for BMP14-induced tenogenic differentiation [84]. Moreover, *SIRT1* was downregulated by TGF-β and identified as a crucial regulator of TGF-β/SMAD signaling in fibroblast activation and tissue fibrosis [85]. In our study, *SIRT1* in BMSCs and teno-iPSC1 was not influenced by any tested tenogenic stimulus, but it was enhanced in teno-iPSC3 under certain conditions, including overexpression of Mohawk, mechanical stretch combined with TGF-β3 or BMP12, indicating that the regulatory network of *SIRT1* gene activity is different among cell types. More studies will be needed to unveil the role of HDACs in the regulation of tenogenic gene expression in stem cells.

5. Conclusions

In summary, our results highlight that both BMSCs and teno-iPSC hold significant tenogenic differentiation capacity. However, the activation of tenogenic genes is highly dependent on the inducers and varies between iPSC clones as well as between cell types. Therefore, additional assessment on the expression of tenocyte-related genes will be needed to achieve the purpose of using predifferentiated stem cells for tendon repair and regeneration.

Data Availability

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

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

FKY and DWR conceived the study, designed the experiments, analyzed the data, and wrote the manuscript. FKY conducted the experiments.

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Supplementary Materials

Supplementary 1. Supplemental Figure 1 Multilineage differentiation capacity of BMSCs. A, B *In vitro* osteogenic differentiation of BMSCs. The calcium deposition was revealed by Alizarin Red S staining. C, D *In vitro* adipogenic differentiation of BMSCs. The fat droplets were displayed by oil-red staining. E, F *In vitro* chondrogenic differentiation of teno-iPSCs. The production of proteoglycan proteins was shown by Alcian blue staining.

Supplementary 2. Supplemental Figure 2 Expression of GFP in lentiviral-infected teno-iPSCs and BMSCs. Teno-iPSCs (A) and BMSCs (B) were infected with lentivirus expressing GFP alone or MKX and GFP for 5 days. GFP signal was imaged under fluorescent microscope.

Supplementary 3. Supplemental Figure 3 Dose effects of TGF-β3 on tenogenic gene expression in iPSC1. iPSC1 were treated with vehicle medium (0) or various concentrations of TGF-β3 (4, 20, and 100 ng/mL) for 5 days, and cDNA was synthesized from total RNA. Expression of tenogenic transcription factors (A), chondrogenic transcription factor Sox9, osteogenic transcription factor RUNX2 (B), and tenocyte-related ECM genes (C) was determined by qPCR. Relative fold change for each group was calculated by comparison to vehicle medium group, and data were summarized from 3 passages. *Data were compared to BSA control; $^\#$data were compared to the 4 ng/mL group; $^\$$data were compared to 20 ng/mL group.

Supplementary 4. Supplemental Figure 4 Dose effects of BMP12 on tenogenic gene expression in iPSC1. Cells were treated with vehicle medium (0) or various concentrations of BMP12 (4, 20, and 100 ng/mL) for 5 days, and cDNA was synthesized from total RNA. Expression of tenogenic transcription factors (A), SOX9, RUNX2 (B), and tenocyte-related ECM genes (C) was determined by qPCR. Relative fold change for each group was calculated by comparison to the vehicle medium group, and data were summarized from 3 passages. *Data were compared to BSA control; $^\#$data were compared to the 4 ng/mL group; $^\$$data were compared to the 20 ng/mL group.

Supplementary 5. Supplemental Figure 5 Sirius red staining in TGF-β3- and BMP12-treated teno-iPSCs and BMSCs. A Cells were treated with TGF-β3 and BMP12 for 5 days, then fixed and stained with Sirius red. B Quantitation of Sirius red staining. $^{*}p < 0.05$; $^{**}p < 0.01$.

Supplementary 6. Supplemental Figure 6 Overexpression of MKX in BMSCs and teno-iPSCs. BMSCs and teno-iPSCs were infected with lentivirus expressing GFP (lenti-GFP) or equine Mohawk and GFP (lenti-MKX) for 5 days, and the whole cell lysates were blotted for MKX and α-tubulin.

Supplementary 7. Supplemental Figure 7 Effects of TGF-β3 or BMP12 on tenogenic gene expression in MKX-overexpressing iPSC1. Cells expressing GFP or equine Mohawk were treated with vehicle medium (GFP/BSA and MKX/BSA), TGF-β3 (20 ng/mL, GFP/TGF-β3 and MKX/TGF-β3), or BMP12 (20 ng/mL, GFP/BMP12 and MKX/BMP12) for 5 days. cDNA was synthesized from total RNA, and expression of tenogenic transcription factors (A), SOX9, RUNX2 (B) and tenocyte-related ECM genes (C) was determined by qPCR. Relative fold change for each group was calculated by comparison to the GFP-CTRL group. *Data were compared to the GFP/BSA group, and $^{\#}$data were compared to the MKX/BSA group.

Supplementary 8. Supplemental Table 1 List of primer pairs used for qRT-PCR in this study.

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Review Article

The Application of Mechanical Stimulations in Tendon Tissue Engineering

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Tendon injury is the most common disease in the musculoskeletal system. The current treatment methods have many limitations, such as poor therapeutic effects, functional loss of donor site, and immune rejection. Tendon tissue engineering provides a new treatment strategy for tendon repair and regeneration. In this review, we made a retrospective analysis of applying mechanical stimulation in tendon tissue engineering, and its potential as a direction of development for future clinical treatment strategies. For this purpose, the following topics are discussed; (1) the context of tendon tissue engineering and mechanical stimulation; (2) the applications of various mechanical stimulations in tendon tissue engineering, as well as their inherent mechanisms; (3) the application of magnetic force and the synergy of mechanical and biochemical stimulation. With this, we aim at clarifying some of the main questions that currently exist in the field of tendon tissue engineering and consequently gain new knowledge that may help in the development of future clinical application of tissue engineering in tendon injury.

1. Introduction

Mechanical stimulus has a huge impact on life activities, which is evident in gene expression, cell life activities, functions of living systems, and individual growth and development. With the redistribution of body fluids and the reduction of skeletal load under weightless conditions, bone loss and increased calcium secretion occur to the bones, which seriously affect the function of the musculoskeletal system [1, 2]. In the context of induced differentiation of stem cells, different types of mechanical stimulations may play different roles. For instance, mechanical stretching has been widely used in tendon tissue engineering to induce tenogenic differentiation, while mechanical compression is beneficial for osteogenic differentiation as well as for chondrogenic differentiation [3–5].

Mechanical stimulation plays a significant role in many aspects of tendon tissue engineering. Applying mechanical stretching to engineered tendons could promote cell infiltration and proliferation [6, 7], induce the extracellular matrix (ECM) deposition and the collagen fiber alignment [6, 8, 9], and also activate mechanically sensitive receptors which subsequently promote tenogenic differentiation [10–12]. In addition, magnetic force could be used as a mechanical stimulus to reduce the formation of fibrous scar tissue and regulate inflammatory responses [13]. Nowadays, mechanical stimulations have been widely used in tendon tissue engineering. However, the optimal regimes of mechanical stimulation for different stem cells for tendon tissue engineering are not yet clarified, nor their inherent mechanisms of mechanical transduction.

In this review, we will make a retrospective analysis of the past decades in the field of applying mechanical stimulation in tendon tissue engineering, as well as the inherent mechanisms. We will also propose some of the most promising directions of mechanical stimulation in tendon tissue engineering. Due to the high similarity in structure and function, tendon and ligament are often discussed undividedly. Therefore, the term tendon is related to both tendon and ligament in this review.

2. Tendon and Tendon Repair

2.1. Tendon Structure. As a highly specialized load-bearing structure, the tendon has an indispensable role in the force transmission between the muscle and bone, thus the tendon is vital for the muscle function and tolerates much higher strain as compared to the muscle belly [14, 15]. Tendons consist of dense regular connective tissues made up of multiple collagen fibrils forming collagen fibers with the paralleled arrangement along the direction of the strain [13]. The tendon tissue ECM is mainly composed of collagen (60%-85% of dry weight) of which approximately 90% is collagen I (COL I) and 10% is collagen III (COL III) and the remaining ECM consist of proteoglycans (1-5% of dry weight). Only few tenocytes, progenitor cells, are located between the collagen fibrils. The sparse vascular supply is either from the related muscle or bone or from the tissue surrounding the tendon, and the nerve innervation is mainly found in the surrounding tissue [15–17]. The hypocellular and the hypovascular natures of tendons determine their poor self-healing capacity after injury [18–20].

2.2. Tendon Repair. Tendon injury is the most widespread musculoskeletal disease, especially the Achilles, patellar, and rotator cuff tendons [20, 21]. Injury to these tendons accounts for more than 30% of all musculoskeletal conditions for which people seek help within the primary healthcare system, and 30 million surgical procedures are performed annually worldwide [22, 23]. Unfortunately, it is almost impossible for tendons to be repaired perfectly due to the poor autonomous healing capability, which frequently results in persistent symptoms and reinjury. At present, tendon injuries are usually treated by conservative or surgical approaches. Conservative treatments include drug injection such as corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs), low-intensity shock wave, ultrasound, and mechanical loading including eccentric training. All these treatment strategies require a long time for the tendon to recover, and the results are often not satisfactory with symptoms that often recur [18, 22, 23]. Surgical treatments such as suture, autograft, allograft, and xenograft also have some disadvantages, including the low availability of grafts, donor site morbidity, infection risk, and inflammatory response [18, 23, 24]. Some biological therapies, such as gene therapy, growth factor therapy, and stem cell therapy, have made great progress over the years by scientists, but most of them are still in the stage of *in vitro* or animal testing; thus, further research is required for subsequent clinical trials [19, 25]. In summary, at present, there is no optimal treatment strategy for tendon injury. New and effective treatments are in urgent need to be developed.

2.3. Tendon Tissue Engineering. Tendon tissue engineering aims at constructing engineered tendons with similar properties to natural tendons and finally to replace damaged tendons by surgery. Commonly, stem cells are seeded in scaffolds and then cultivated in an environment with appropriate growth factors and/or biomechanical stimulation, aiming at constructing an ideal tissue-engineered tendon. Based on the principles of tissue engineering, there are three strategies to optimize the engineered tendon: selecting the ideal cells, improving the properties of the scaffold, and providing appropriate growth factors and/or biomechanical stimulation [26]. Various stem cells have been applied in tendon tissue engineering. For instance, bone marrow mesenchymal stem cells (BMSCs) [27, 28], adipose stem cells (ASCs) [29], and tendon-derived stem cells (TDSCs) [6, 10, 15]. The cell type most commonly applied for tissue engineering is BMSCs, which are multipotent stem cells isolated from bone marrow with self-renewal capability, multilineage differentiation potential, and immune system tolerability [30, 31]. ASCs are stem cells derived from adipose tissue with fantastic differentiation and migration capacities, and they seem to be quite appropriate for tendon therapy [32]. TDSCs have been isolated from tendons of different organisms, and they have similar self-renewal and multilineage differentiation capacities as the BMSCs but have a higher expression level of tendon-related genes [6]. Therefore, TDSCs are potentially the ideal cells to use for tendon tissue engineering. Nowadays, synthetic materials like polylactic acid and polyglycolic acid have been applied to create scaffolds for tendon tissue engineering [6, 33, 34]. However, researchers are also keen to find out the excellent biomaterials, such as collagen [35, 36], silk fibroin [36], alginate, and gelatin [23]. In addition, the structure of the scaffolds is also important to optimize. It is known that a few hundred-micron pore sizes and a porosity of over 90% in the scaffold material facilitate cell infiltration [37]. Scaffolds made of aligned fibers with wavy morphology exhibit excellent mechanical properties and the effects in promoting cell proliferation, infiltration of cells in-between the fibers, and stimulating tenogenic differentiation [38–41]. In addition, various growth factors have also been shown to stimulate tenogenic differentiation and tendon regeneration such as connective tissue growth factors (CTGF), transforming growth factors β (TGF-β), and growth differentiation factors (GDF) [42]. Although many studies on tendon tissue engineering have been conducted in the past decades, there still exist some major challenges, such as the comprehensive consideration of the mechanical properties of the scaffold and its integration with cells. Optimal biomechanical stimulation during tendon tissue engineering may improve the construct by regulating the remodeling of ECM and promotion of cell infiltration [7], alignment [6], proliferation [7], and differentiation [35].

3. Main Strategy of Mechanical Loading in Tendon Tissue Engineering

Biomechanical signals are involved in the growth and development of organisms, which can stimulate and induce tissue formation. Biomechanics is a branch of biophysics, which applies the principles and methods of mechanics to the quantitative research of biomechanical problems in for example blood, body fluids, organs, and bones. The physiological mechanical stimulus on the tendon is comprised of tensile strain, shear force, and compression (Figure 1) [10]. Due to the main function of tendon, the mechanical stretching caused by tension is the main mechanical stimulus

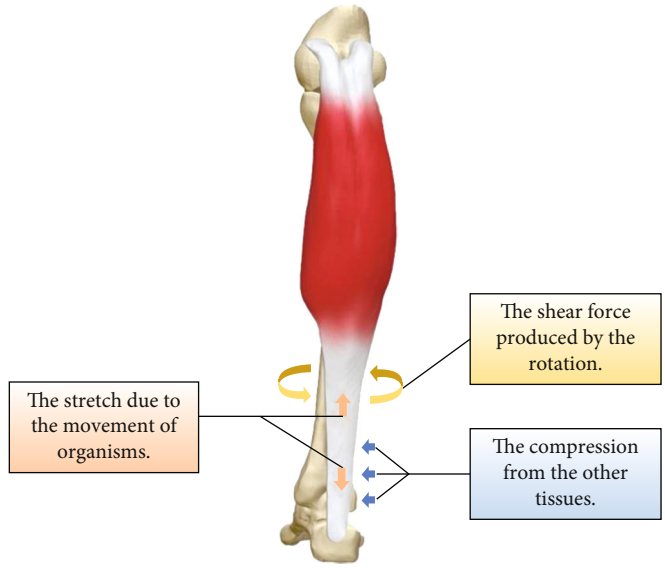

Figure 1: Biomechanical stimulations in natural tendon. Three arrows with different colors indicate three types of biomechanical stimulations including stretch, shear force, and compression.

throughout the growth and development of tendons. Therefore, it is reasonable to provide mechanical stretching for the construction of engineered tendons to mimic the natural microenvironment of tendons. In tendon tissue engineering, dynamic and static stretching is currently the most widely used mechanical stimulation *in vitro*[11, 20, 43]. Natural mechanical stimulation *in vivo* has also been applied to tendon tissue engineering with great significance.

3.1. Dynamic and Static Stimulation In Vitro. Mechanical stretching is the main strategy to achieve mechanical loading in tendon tissue engineering, which could be divided into dynamic and static mechanical stretching [11, 20, 43]. Appropriate mechanical stretching is beneficial to the formation of engineered tendons as it regulates cell behaviors and tissue remodeling [6, 7, 24, 35]. However, mechanical stretching can also have negative effects. For instance, mechanical stretching may increase the diameter of the scaffold, make it elongated, and decrease its Young's moduli [23, 35], i.e., reducing the mechanical properties of the scaffold. Also, excessive mechanical stretching will result in early differentiation and apoptosis of stem cells [23, 43]. The applications of mechanical stretching during the past decades are summarized in Table 1.

Dynamic stretching is the most commonly used type of mechanical stretching stimulus and can be regulated by three main parameters, which are (1) strain, (2) frequency, and (3) rest interval [11]. Different protocols usually bring different and even opposite effects to tenogenic differentiation and tendon tissue engineering.

(1) Strain. It has been reported that mechanical stretching can not only induce tenogenic differentiation but also promote osteogenesis, adipogenesis, and chondrogenesis, which is closely related to the percentage of strain [10, 18, 59]. Thus, it could be speculated that only mechanical stretching of a certain range of strain can induce tenogenic differentiation. However, researchers have reached different, or even opposite conclusions. For example, Chen et al. found that the lower strain (3%) promoted osteogenic differentiation, while the higher strain (10%) upregulated the expression of tendon and ligament-related genes [18]. However, Patel et al. found that a 4% strain promoted the tendon differentiation of TDSC, while an 8% strain might induce osteogenesis, adipogenesis, and chondrogenesis [59]. Actually, the reported strains that could promote tendon differentiation range from 1% to 15% (Table 1). According to the physiological strain of the tendon *in vivo*, the strain of dynamic stretching should be 4%-8% (at most 10%) [60]. Zhang et al. and Rinoldi et al. both applied dynamic stretching with a 15% strain in their studies. Although it promoted expression of tendon-related genes such as *Scleraxis* (*SCX*) and *Tenascin-C* (*TNC*), it resulted in lower protein expression of COL I and TNC [23, 35]. In addition, Nam et al. found that human BMSCs had the highest expression of tendon-related genes and proteins at strain conditions of 8% and 12%, and the latter reached its peak faster [27, 28]. Therefore, a strain of 1%–12% seems to be a broad range that could be applied in tendon tissue engineering. Generally, a too high strain may cause early cell differentiation and apoptosis and may also reduce the mechanical properties of the scaffold, such as excessive elongation or increased pore size [23, 35]. On the other hand, a too low strain may not have the expected stimulatory effects. Due to different loading methods and loading systems (such as duration, tissue fixation methods, and stem cell types), the optimal strain varies and should thus be optimized for each specific condition [18]

Table 1: Dynamic uniaxial stretching used in tendon tissue engineering.

Cell type	Parameters	Effects	Ref
Human BMSCs	1% strain; 1 Hz; 30 min/day.	Maintained the expression of SCX.	[44]
Rat BMSCs	2% strain; 0.5 cycles/min; 30 min/day.	Increased cellularity and tensile strength; promoted ECM deposition and fiber alignment.	[24]
Rat BMSCs	2% strain; 0.5, 1, and 2 cycles/min; 0.5, 1, and 2 h/day.	Significantly increased cellularity and tensile strength; further ECM deposition and fiber alignment.	[43]
Rabbit TDSCs	2% strain; 1 Hz; stretching and rest alternated.	Promoted tenogenic differentiation (COL 3A1 and DCN).	[37]
Rat BMSCs	2.4% strain; 1 Hz; stretching for 20 s and resting for 100 s.	Significantly promoted *COL I* expression; increased stiffness of construct.	[45]
Rabbit MSCs	2.4% strain; 1 Hz, 8 h/day.	Significantly increased *COL I* expression. Increased the linear stiffness of construct.	[46]
Dog BMSCs	3.0% strain; 0.2 Hz; 20 min/h, 12 h/day.	The elongated cell morphology; promoted cell infiltration and retained mechanical properties; promoted the tenogenic differentiation.	[7, 47, 48]
Equine BMSCs, ASCs, TDSCs	3% strain; 0.33 Hz; 1 h/day.	Promoted cell infiltration and tenogenic differentiation; increased mechanical properties.	[49]
Human ASCs	4% strain; 0.5 Hz; 2 h/day.	Significantly increased the tendon-related genes and proteins.	[33]
Rabbit BMSCs	A 5% translational strain and a 90° rotational strain; 0.1 Hz; 12 h/day.	Upregulated the expression of tendon-related ECM proteins (COL I, TNC, and TNMD); promoted cell alignment.	[50]
Human BMSCs	5% strain; 1 Hz; 1 h/day.	An upregulation in a number of key tendon genes (*Col1a1*, *Col1a2*, *Col3a1*, *TNC*, *ELN*, and *FN*).	[51]
Rat TDSCs	6% strain; 0.25 Hz; 8 h/day.	Induced tenogenic-specific differentiation; aligned and compact F-actin network.	[10]
Human fibroblasts	10% strain; 0.25 Hz; 8 h/day.	Significant increased cell proliferation and increased *COL I*, *TFG-β1*, and *CTGF* expression; increased COL I and FN deposition.	[52]
Human BMSCs	10% strain; 0.33 Hz.	Increased *COL I*, *COL III*, and *SCX* expression compared to control group.	[53]
Murine fibroblasts	10% strain; 0.5 Hz.	Better alignment of collagen fibers and proper organization of ECM.	[15]
Human BMSCs	10% strain; 1 Hz; 2 h/day.	Enhanced expression of *COL I*, *EphA4*, and *SCX*; elongated cell morphology.	[34, 54]
Human BMSCs	10% strain; 1 Hz; 3 h of strain followed by 3 h rest.	Significantly upregulated tendon related genes (*COL I*, *COL III*, and *TNC*).	[55]
Human BMSCs	10% strain and axial rotation; 1 Hz.	Significantly enhanced cell infiltration, matrix synthesis (COL I and III and TNC), and ultimate tensile load of engineered tendons.	[8]
Equine BMSCs	3%, or 5% strain; 0.33 Hz; 1 h/day.	3% strain promoted cell infiltration, tenogenic differentiation, and increased construct elastic modulus and ultimate tensile strength.	[56]
Human BMSCs	4, 8 or 12% strain; 0.5 or 1 Hz.	The highest proliferation rate at 1 Hz and at 4% strain. The highest tenogenic expression at 8% and 12% strain.	[27]
Human BMSCs	4, 8 or 12% strain; 1 Hz.	Higher tenogenic gene expressions at 8% (highest) and 12% strain (COL I, COL III, FN, and N-cadherin).	[28]
Rabbit TDSCs	*In vitro*: 4% strain; 0.5 Hz; 2 h/day. *In vivo*: implanted into the mouse back.	*In vitro*: promoted tendon-specific genes and protein expression. *In vivo*: more parallelly arranged matrixes (COL I, COL III, and TNC); the mature engineered tendon. Both: increased cell proliferation, elongated cell morphology, and mechanical properties.	[6]
Human ESC-MSCs	*In vitro*: 10% strain; 1 Hz; 2 h/day. *In vivo*: implanted into the mouse back.	*In vitro*: unregulated the expression of tendon-related genes *(SCX*, *COL I*, *COL III*, and *Epha4*). *In vivo*: elongated morphology of cells; promote more parallel alignment.	[57] [58]
Rat BMSCs	*In vitro*: 15% strain; 1 Hz. *In vivo*: implanted into the hind limbs of mice.	*In vitro*: increased cell viability and the expression of *SCX and TNC*; COL1a1 and TNC expression did not significantly change; increased pore size. *In vivo*: better mechanical properties and cell alignment (after prestretching *in vitro*).	[35]

(2) Frequency. Most of the stretching frequency applied in tendon tissue engineering is not higher than 1 Hz (Table 1). Some studies found that 1 Hz of mechanical stretching may be the best condition to induce various cellular responses including a high level of cell proliferation and tenogenic differentiation [11, 27]. However, Engebretson et al. demonstrated that the lower frequencies are better for improving the quality of engineered tendons, while the positive effect of stimulus would decrease when it is over 1 cycle/min (0.017 Hz). They found that the lower frequency and shorter duration (1 cycle/min and 0.5 or 1 h/day) were more likely to promote the production and alignment of COL I fibers and cell proliferation as compared to higher frequency and longer duration [43]. Generally, lower frequencies (below 1 Hz) are beneficial for cell proliferation and tenogenic differentiation. The optimal stretching frequency from each study is different, probably due to the differences in bioreactors, cell types, and other stretching parameters. Mechanical stretching with higher frequency (higher than 1 Hz) influences cell proliferation and reduces the expression of ECM proteins in tendons [23, 43], and it may also induce apoptosis, which can explain why cell proliferation decreases [43]

(3) Rest Interval. Cells gradually adapt to the stimulus, thereby a reduction in the effect of applied mechanical stimulus [11]. By adding rest interval, the mechanical sensitivity of the cells can be restored, and ultimately more positive effect can be achieved [61]. As mentioned earlier, Engebretson et al. found that groups with shorter duration and lower frequency had higher levels of cell proliferation. The highest proliferation was found in the group with mechanical stretching 0.5 hour/day and with 1 cycle/minute, which resulted in an increase of 203% as compared to the static control. Mechanical stretching lasting longer than 1 hour/day would limit its beneficial effects due to adaptation to the stimulus [43]

In general, the effects of various parameters (range of strain, frequency, rest, and duration) of dynamic stretching on tenogenic differentiation are significant but it is difficult to distinguish whose impact is most efficient. To evaluate the role of each parameter of dynamic stretching in tendon tissue engineering, a bioreactor capable of regulating different parameters at the same time is essential. A bioreactor can provide suitable biomechanical and biochemical stimulus to the engineered tendon constructs, mimicking the microenvironment of natural tendons. The activating system and the culture chamber are the main components of bioreactors. In addition, other systems can be added to achieve circulation and in-depth analysis of the medium. Presently, the LigaGen system (http://www.tissuegrowth.com) and the Bose® ElectroForce® BioDynamic® system (http://www.bose-electroforce.com) are the well-developed commercially available bioreactor systems. Both systems can provide precise and programmed mechanical stretching. The LigaGen system can detect the stiffness of the sample in real-time and adjust the instrument itself according to different requirements. The Bose® ElectroForce® BioDynamic® system can monitor sample strain and perform biomechanical tests in real-time [62]. In addition, various bioreactors are also developed by different research groups to meet their own individual specific requirements, and some of them showed a good performance in constructing engineered tendons (Figure 2) [58, 60, 63].

Some earlier studies also reported the positive effect of static mechanical stimulation on tenogenic differentiation (Table 2). Dynamic stretching with higher frequency and longer duration decreases the level of cell proliferation [43]; comparably, it was found that continuous application of static stretching will reduce the mechanical sensitivity and thus the proliferation of cells. In addition, long duration of static stretching will reduce the total tension to the cells for two reasons; (1) due to adaptation to the stimulus and (2) all newborn cells will not sense the stretching [23]. Therefore, currently, more and more studies in this field have focused on the optimization of protocols for dynamic stretching, instead of static mechanical stimulation.

3.2. 2D and 3D Loading Models In Vitro. At present, two-dimensional (2D) loading models and three-dimensional (3D) loading models *in vitro* have been applied to the research of tendon mechanobiology. In 2D loading models, cells are usually seeded on a sheet and receive mechanical stretching indirectly by stretching the sheet [10]. In this model, mechanical stretching can be accurately transmitted to the cytoskeleton, and the relationship of biological response and mechanical stimulation can be studied [68]. Both uniaxial and biaxial stretching have been applied to 2D loading models and have been shown to influence the promotion of tenogenic differentiation [68]. However, there are some disagreements regarding stem cell differentiation with the application of biaxial stretching. Wang et al. found that uniaxial loading promoted tenogenic differentiation but biaxial loading induced chondrogenic, adipogenic, and osteogenic differentiation of TDSCs [10]. However, some other researchers have shown that biaxial loading could also promote tenogenic differentiation [8, 69–71]. Biaxial loading provides multidirectional stretching, including longitudinal and transverse or circumferential directions, which is different from the physiological mechanical environment of tendon cells [68]. Therefore, stem cells may simultaneously show the higher expression of multiple tissue genes under biaxial stimulation, and the differences in gene expression levels may be caused by different loading conditions and cell types.

Even though the 2D loading model can be used to investigate the effects of mechanical stimulus on cells, it cannot replace the significance of 3D loading models since the effect of mechanical stretching on 3D engineered tendon constructs are influenced by many factors such as pore size, topography, and the material of the scaffolds [68]. The 3D loading models are constructed by seeding cells in a 3D material, which transfers stretching to the embedded cells. The effect and involved signaling pathways of the same stretching protocol could differ dramatically between a 2D loading system and a 3D loading system. For instance, Wang et al. found that osteogenesis and adipogenesis differentiation was

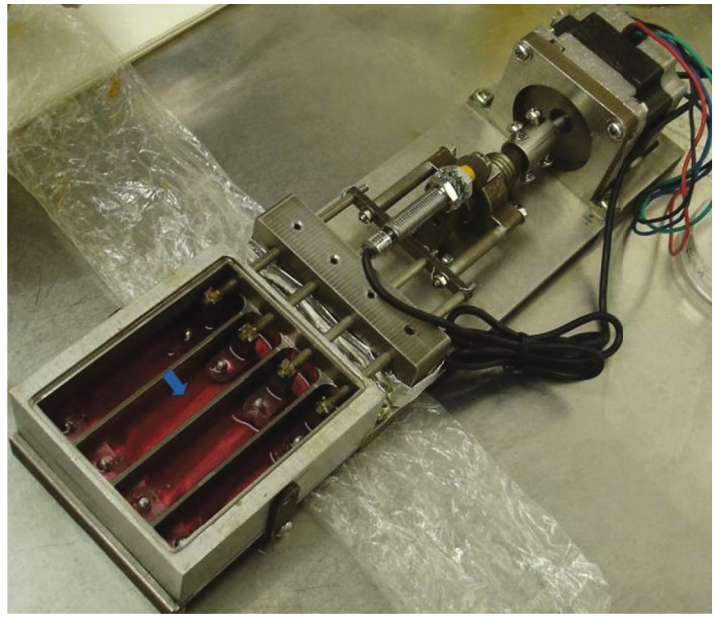

Figure 2: A custom-made bioreactor. Engineered tendon (cell-seeded scaffolds) is placed within each chamber (blue arrow), with two ends anchored. Amplitude and frequency of the bioreactor could be set.

Table 2: Static uniaxial stretching used in tendon tissue engineering.

Cell type	Parameters	Effects	Ref
Equine ASCs	4% strain; stretching for 2 h and followed by a 6 h pause.	Promoted cell alignment; more spindle-shaped cell and the elongated nucleus.	[64]
Human BMSCs	15% strain (day 0-7); 30% strain (after 7 days).	Enhanced COL I and III expression and its alignment; promoted *SCX*, *TNMD* expression. Promoted cell adhesion, alignment, and proliferation.	[23]
Human dental pulp stem cells (DPSCs)	Maximum tensile force (just below the failure load).	Expressed *COL I* and *VI* but rarely expressed tendon-related proteins.	[65]
Human BMSCs	Double the length of the construct.	The packed and aligned fibrils. Increased ultimate tensile stress.	[66]
Human BMSCs	The constant tension generated by a bioreactor.	Upregulated the expression of SCX; modulated elastin and COL III, XII, and XIV expressions.	[44]
Human fibroblasts	The constant tension generated by a U-shaped spring.	Production of fibers of COL I & III that were aligned longitudinally.	[67]

promoted by 2D uniaxial loading, but inhibited by 3D uniaxial loading using the same mechanical stretching protocol [10]. Connexin 43 is a gap junction protein that mediates intercellular communication. Wang et al. discovered that 2D-loaded cells expressed more connexin 43 when uniaxially loaded; however, the opposite results were obtained in 3D loaded tendons. This indicates that under 2D conditions, cells perceive mechanical stimulation through cell body junctions, while under 3D conditions, cells perceive mechanical stimuli through cell-ECM interactions. It has been found that mechanical loading under 3D conditions can promote tenogenic differentiation and tendon ECM remodeling which facilitate the construction of engineered tendon[6, 11, 23, 24]. Compared with the 2D loading system, the 3D system more closely simulates the physiological mechanical loading of natural tendons. Therefore, the 3D loading model is more relevant for tendon tissue engineering.

3.3. Natural Mechanical Stimulation In Vivo. The long-term goal is that tissue-engineered tendons eventually will be used clinically; therefore, its construction and functionality *in vivo* are of great significance. Some groups transplanted engineered tendons into the knee joints [8, 57] and backs [58] of miniature pigs or mice to give them physiological mechanical stimulation (Figure 3), which is caused by the movement of the recipient animals. These natural mechanical stimulations induced the formation of a more mature tendon-like tissue by promoting the tenogenic differentiation of stem cells, inducing a physiological cell shape and arrangement, and promoting the deposition and arrangement of tendon ECM [6, 57, 58]. Juncosa-Melvin et al. showed that the maximum stress of engineered tendons cultured *in vivo* increased by 3000 times after 2 weeks, which could not be accomplished in any current bioreactors *in vitro* [62, 72]. Therefore, the natural mechanical stimulation has great potential if

Figure 3: Engineered tendon transplanted into the back of a nude mouse to exert physiological mechanical stimulation. The construct is sutured to the fascia, which receives mechanical stretching caused by the natural movement of the mouse back. Reprinted from Biomaterials (2010), Vol. 31, Chen JL, et al., Efficacy of hESC-MSCs in knitted silk-collagen scaffold for tendon tissue engineering and their roles, Pages 9438-9451, Elsevier (2010), with permission from Elsevier.

applied to tendon tissue engineering. Interestingly, Zhang et al. and Xu et al. prestretched engineered tendon *in vitro* before applying natural mechanical stimulation *in vivo*. The prestretched group was found to have more deposition of aligned tendon ECM, as compared to the unstretched [6, 35]. The reason for the positive effect might be because the prestretching *in vitro* promotes the integration of seeded cells and the scaffold, thus show better stimulatory effects after transplantation. In conclusion, natural mechanical stimulation and the combination of mechanical stretching *in vitro* and *vivo* help the formation of matured engineered tendons, which are of great significance for tendon tissue engineering.

4. Effect of Mechanical Loading on Tendon Tissue Engineering

Mechanical stimulation has been shown to promote the tenogenic differentiation of stem cells and the deposition of tendon ECM, thus improving the properties of engineered tendon constructs. Until now, different mechanical stimulation protocols have been reported in tendon tissue engineering to mimic the mechanical environment of tendons under natural conditions. Some previous studies have demonstrated that mechanically stimulated tissue-engineered tendon shows more promising results in tendon repair and regeneration *in vivo*. For instance, Xu et al. evaluated the repair effect of mechanical stimulated engineered tendons in a rabbit patellar tendon defect model. They found that the repaired tendons in the experimental group exhibited more and aligned collagen fibers, aligned spindle-shaped healing tenocytes, and significantly increased ultimate stress and Young's modulus, as compared to those in the control group [6]. Lee et al. applied the mechanically stimulated engineered tendon to porcine anterior cruciate ligament (ACL) reconstruction *in vivo* and found that the ultimate tensile load of the repaired tendons improved significantly (within 80% of the native porcine ACL) after three months postsurgery, with higher matrix synthesis and increased stiffness, as compared to the tendons repaired by the nonmechanical-stimulated engineered tendon [8]. Furthermore, some studies discovered that engineering tendon with mechanical loading *in vivo* exhibited more mature collagen fibrils, better-aligned collagen fibers, and bigger tissue volume with improved mechanical properties, as compared to the loading *in vitro* and the nonloading *in vivo*[6, 73, 74]. In general, there are two explanations of how mechanical stimulation promotes tendon repair and regeneration.

(1) Mechanical stimulation promotes the tenogenic differentiation of stem cells. The gene expression of tendon markers and the synthesis of tendon ECM are two important outcomes to measure tenogenic differentiation. As tendons have no specific markers, expressions of several important tendon-related markers are usually detected, such as Scleraxis (SCX), Mohawk (MKX), and Tenomodulin (TNMD) [9, 23]. SCX is a known early transcription factor expressed in tendon progenitor cells and tenocytes [75, 76]. MKX is recognized as a transcription factor expressed in developing tendons [76]. And TNMD is a tension-regulating protein, which is related to the phenotype of tenocytes and is considered as a late marker of tendon formation [7]. As shown in Tables 1 and 2, mechanical stimulation can promote the expression of these important tendon-related markers. COL I is the main component of tendon ECM, therefore promoting the expression of COL I can be considered, to some extent, as a marker of tenogenic differentiation of stem cells. In addition, other ECM molecules such as COL III, decorin (DCN), Tenascin-C (TNC), N-cadherin, elastin (ELN), and fibronectin (FN) are other components of the tendon, and therefore their expressions were evaluated as well in many reports [7, 28, 51]. However, all those ECM molecules could also be found in many other tissues. For example, the tissues expressing COL I include tendons, basement membranes, and skin and blood vessels [77]. Based on the fact that there is not any unique ECM molecule only found in tendons, it is not enough to evaluate

tenogenic differentiation by only using the expression of certain ECM molecule

(2) Mechanical stimulation regulates cell behaviors and improves the mechanical properties of engineered tendons by remodeling of the ECM. It has been found that mechanical stimulation promotes cell proliferation [6, 24], migration [23], infiltration [6, 8], and alignment [6], as well as ECM deposition [7, 24], which are all of great significance for successful construction of engineered tendons. For instance, Xu et al. cultured the TDSC-seeded poly(L-lactide-co-ε-caprolactone)/collagen construct under dynamic stretching for tendon tissue engineering. They found that mechanical stimulation induced an increased proliferation and a similar morphology with tenocytes, and finally it increased the expression of tendon-related ECM genes and proteins, which resulted in significantly improved mechanical properties of the engineered tendon (about 52% of Young's modulus and 60% of ultimate tensile stress of the nature tendon) [6]

5. Signal Transduction of Mechanical Loading in Tendon Tissue Engineering

Substantial progress has been made in the study of signal transduction following mechanical stimulation. Cells sense and deliver mechanical stimulation through cell adhesion molecules (CAMs) [78]. CAMs are dynamically connected to the cytoskeleton, responding to mechanical tension and transmitting stimulus to the nuclear membrane, which in turn triggers changes in cellular gene expression [78]. Since there are only a few cells in both natural and engineered tendons, the transmission of mechanical stimulus in tendon tissue engineering is more likely to depend on the interaction between cells and ECM by CAMs [10], which is a mechanism that should be further explored in order to construct engineered tendons successfully.

It has been reported that the signal transduction network consists of focal adhesion kinase (FAK) [11, 12], phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) [10, 79], Rho proteins/Rho-associated protein kinase (RhoA/ROCK) [12], yes-associated protein/transcriptional coactivator with PDZ binding (YAP/TAZ) [13], and Smad [10, 80]. All of these signal transducers are involved in tenogenic differentiation induced by mechanical stimulation (Figure 4). There are also some mechanically sensitive receptors on the cell membrane, such as integrins, growth factor receptors, and stretch-activated ion channels [78]. Transmembrane integrins connect ECM proteins and focal adhesion proteins, and the latter are connected to the nuclear membrane through the cytoskeleton (actin fibers). Thus, integrins can transmit forces across the nuclear membrane and mediate the response of mechanically loaded cells [13]. Besides, integrins can also detect the stiffness (elasticity), topography, and surface chemistry of the matrix [81]. These detections by the integrins can activate FAK and RhoA and thereby induce changes in downstream signal molecules [81]. PI3K/AKT pathway has been demonstrated to participate in the regulation of tenogenic differentiation as the downstream pathway of FAK [10, 79, 81]. For example, Wang et al. and Cong et al. found that when the PI3K/AKT pathway was inhibited, tenogenic differentiation and the formation of engineered tendons are weakened [10, 79]. Therefore, although the PI3K/AKT pathway has also been reported to be associated with osteogenic differentiation of stem cells, it does play an important role in the tenogenic differentiation induced by mechanical stimulation. RhoA/ROCK pathway is another downstream signaling pathway of mechanical loading. RhoA is a member of the small G protein superfamily and can activate downstream ROCK. RhoA/ROCK is a downstream molecule of integrins, which together with FAK regulates mechanical stretch-induced cytoskeletal reorganization [81]. Xu et al. discovered that RhoA/ROCK affected FAK activation and coregulated the formation and rearrangement of actin fibers, thereby inducing tenogenic differentiation. At the same time, the cytoskeleton appeared to regulate its changes through feedback [12]. Besides, Tomás et al. demonstrated that changes in cytoskeleton tension following mechanical stimulus can activate YAP/TAZ in the cytoplasm to be transferred into the nucleus and promote the expression of tendon markers *SCX* and *TNMD* [13]. The TGF-β/Smad pathway is recognized as the most relevant pathway to tendon differentiation [82, 83]. It has been reported that mechanical stimulation and growth factors like TGF-β and BMP-12 (GDF-7) can activate growth factor receptors such as the TGF-β type I/II receptor. These receptors can contribute to the activation of the downstream Smad 2/3/8 pathway which promotes tendon formation [10, 80]. In addition, mechanical stimulation can activate mechanically sensitive ion channels, leading to the influx of cations (such as Ca^{2+}), thereby inducing some cellular responses including the transmission of intracellular signal, actin polymerization, and cytoskeletal remodeling [11, 78, 84].

6. Promising Directions of Mechanical Stimulation in Tendon Tissue Engineering

6.1. Magnetic Force Stimulation. A previous study has demonstrated that magnetic stimulation has improved the biological performance as compared to the equivalent nonmagnetic mechanical stimulation in tendon tissue engineering [13]. Magnetic stimulation usually includes two aspects: the action of the magnetic field and the indirect mechanical force produced by a magnetic field upon magnetic particles (MNPs).

A low-frequency magnetic field has been applied to regulate the inflammatory response in tendon treatment [84]. Furthermore, Pesqueira et al. demonstrated that low-frequency static magnetic field promoted the expression of tendon-related genes (*SCX*, *COL1A1*, *COLA3*, *TNC*, and *DCN*) by regulating the intracellular calcium ion concentration and activation of oxygen release, and that the effect was related to the duration of exposure [84]. Hence, applying magnetic field in tendon tissue engineering has great potential.

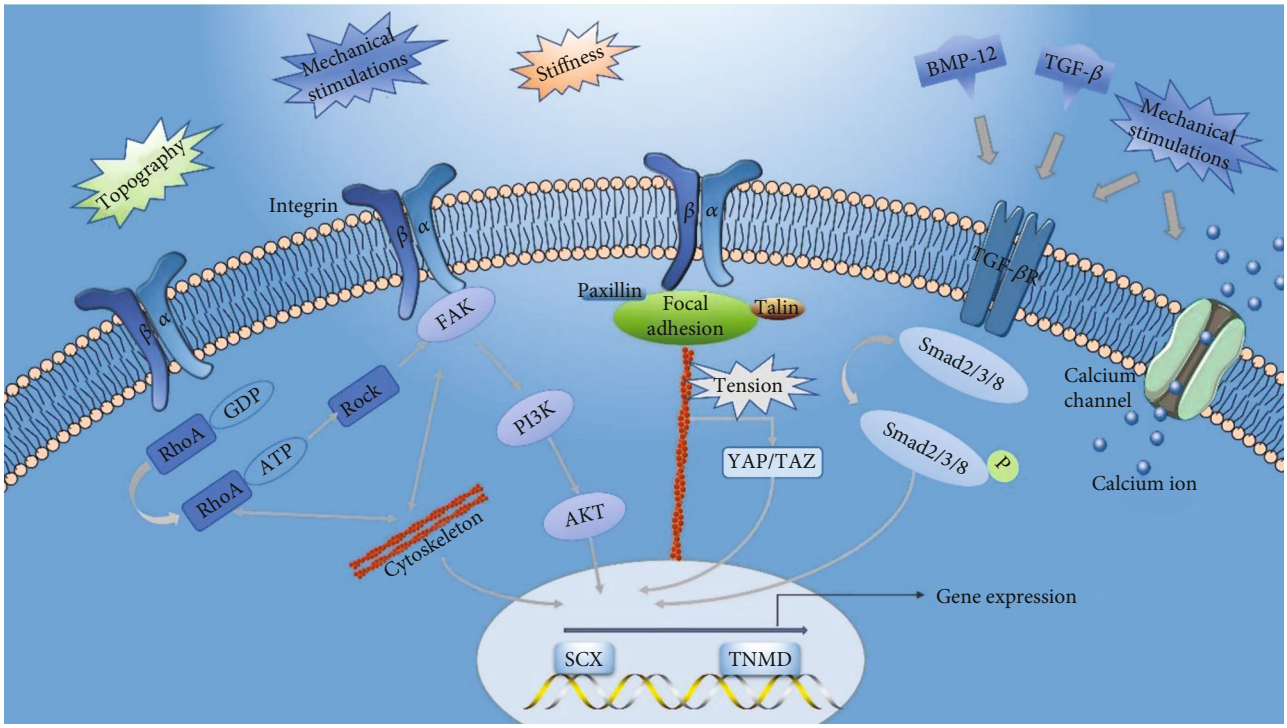

FIGURE 4: Signal transduction of mechanical loading in tenogenic differentiation. The mechanical stimulations and the mechanical properties of substrate including topography and stiffness can activate the integrins, which causes the changes of downstream signaling molecules and results in tenogenic differentiation. The mechanical stimulations and growth factors both can activate the TGF-β receptors, inducing tenogenic differentiation. The mechanical stimulations can open the calcium channel and bring about the inflow of Ca^{2+}, which regulates tenogenic differentiation.

Interestingly, the studies of magnetic force upon MNPs also have shown promising results in tendon tissue engineering [13, 80, 85, 86]. In biomedicine, MNPs have been used to label, track, and promote the life activities of stem cells such as proliferation, migration, and differentiation [86]. MNPs have two applications in tendon tissue engineering. (1) The direct application of MNPs alone. MNPs can be cocultured with stem cells. The mechanical force generated by the magnetic field will be transmitted to the stem cells, thus promoting their tenogenic differentiation. Gonçalves et al. labeled the human ASCs with chitosan-encapsulated MNPs to construct magnetically functionalized cells, which can be subjected to indirect and adjustable mechanical stimulation by applying a magnetic field [85]. In another study, they attached MNPs to antibodies (activin), which made the MNPs specifically bind to the RctRIIA (mechanically sensitive receptor). When supplied with a suitable magnetic field, the RctRIIA was remotely activated, which resulted in the activation of the Smad 2/3 pathway and triggered a tendon-related transcription response [80]. Both of these attempts can effectively deliver mechanical stimulation to cells and induce tenogenic differentiation without relying on scaffolds, which therefore avoids the possible negative effects of mechanical stimulation on scaffold properties such as increased pore size, elongation, and decreased elasticity. In addition, it can provide regular mechanical stimulation for the engineered tendon after transplantation, which may play a positive role in promoting tendon repair and regeneration. (2) The application of MNPs incorporated scaffolds. MNPs can be used to fabricate magnetically responsive scaffolds. MNPs in the scaffold can vibrate in response to an external magnetic field, which deflects the material to produce a transient physical force. This force can be transferred to the cells embedded in the scaffold, driving the tenogenic differentiation of the stem cells [13, 86]. Tomás et al. applied this strategy to cultivate engineered tendons under a magnetic field of 1.5 mT, and observed high expression of *SCX* and *TNMD* while the genes of other lineages were suppressed [13]. The upregulation of anti-inflammatory markers was also found during the process [13].

In summary, magnetic force stimulation has several advantages compared with other types of mechanical stimulation. (1) Magnetic force stimulation can regulate the inflammatory response, and therefore obtain the engineered tendon with better biological performance. (2) Magnetic force stimulation can be remotely and easily adjusted by changing the magnetic field, even *in vivo*. (3) Through MNPs, or target activated receptors, magnetic force stimulation can more effectively deliver mechanical stimulation to seeded cells.

6.2. The Combination of Mechanical and Chemical Stimulations. Various growth factors have been widely applied to tendon tissue engineering, and some of them have been shown to promote tenogenic differentiation and tendon regeneration. For instance, the signal pathway mediated by

Table 3: Combination of the mechanical and biochemical stimulations in tendon tissue engineering.

Cell type	Biochemical stimulation	Mechanical stimulation	Effects	Ref
Murine fibroblasts	5 ng/ml TGF-β1.	10% strain; 0.5 Hz.	Synergistically promoted the tenogenic differentiation.	[15]
Rat BMSCs	10 ng/ml TFG-β1.	15% strain; 1 Hz.	Synergistically increased cell viability, the tenogenic differentiation, and the mechanical properties of construct.	[35]
Human BMSCs	10 ng/ml BMP-12.	Static tension (day 0~7: 15% strain; day 7~: 30%)	Synergistically promoted the tenogenic differentiation and cell alignment.	[23]
Human BMSCs	hGDF-5/BMP-14 (loaded into the PLGA microcarriers).	10% strain; 1 Hz.	Synergistically induced the expression of COL I and III, DCN, SCX, and TNC.	[34]
Equine ASCs	10 ng/μl GDF-5,6,7.	4% strain; stretching for 2 h; followed by a 6 h pause.	Induced the higher tendon associated gene expression, especially for COMP and SCX compared single stimulus.	[64]
Rat BMSCs	1600 ng/scaffold GDF-5.	10% strain; 0.33 Hz.	Mechanical stimulation and GDF-5 increased the expression of *COL I* and *SCX* compared to control. No obvious additive synergism.	[53]

TGF-β1 is considered to be the most important signal transduction pathway that induces tenogenic differentiation. CTGF contributes to the structural integrity of tendon tissue [42]. In addition, GDF-5/6/7 has been reported to promote the expression of tendon-related genes [87–89]. Researchers usually discuss the effects of mechanical stimulations and growth factors on tenogenic differentiation separately, but each of them alone is not sufficient to get a satisfactory engineered tendon. Some growth factors are important for tendon repair and regeneration, even throughout the entire process, such as TGF-β [15]. We cannot guarantee that mechanical stimulation will replace the effect of growth factors or that mechanical stimulation will induce similar effects as growth factor. However, mechanical stimulation can be a good supplement to growth factors, especially for the promotion of tendon formation. It might also be that mechanical stimulation will affect the expression pattern of receptors on the cells, thus increasing or decreasing the response of certain growth factors. For example, the decreased mechanical loading downregulates the expression of TGF-β receptors and thus suppresses the TGF-β/Smad signaling pathway which is significant for tenogenic differentiation [90]. Therefore, it is of great importance to explore the synergistic combination of mechanical stimulation and growth factors.

There are a few studies that have demonstrated the synergistic effect of mechanical stimulation and growth factors like TGF-β1 and BMP-12 (GDF-7) in tendon tissue engineering (Table 3). Zhang et al. discovered that dynamic stretching and TGF-β1 synergistically increased cell viability and the expression of tendon-related genes (*Col 1a1*, *Col 3a1*, *TNC*, *SCX*, and *TNMD*) as well as their corresponding proteins [35]. Interestingly, their synergistic effects are not manifested in copromotion, but instead seem to make up for each other's negative effects. For instance, Zhang et al. found that the dynamic stretching could suppress the cell death induced by TGF-β1 and that growth factor inhibited the increase of the average porosity and pore size caused by cyclic stretching, which improved the mechanical properties of the engineered tendon [35]. Testa et al. demonstrated that the combination of biochemical and mechanical stimulation could synergistically promote tenogenic differentiation, resulting in an abundant and aligned type I collagen [15]. Besides, Rinoldi et al. constructed the cell-loaded highly aligned hydrogel yarns and cultured them under 15% static stretching during simultaneous exposure of BMP-12 for 7 days. They found that the synergistic action promoted the upregulation of *SCX* and *TNMD*, inducing the tenogenic differentiation of human BMSCs. Nevertheless, they also found that the expression of COL I and III were inhibited, which seemed to be related to the early differentiation and apoptosis caused by excessive strain [23]. However, not all the combinations of mechanical stimulation and growth factors can synergistically promote tendon differentiation. Farng et al. cultured the engineered tendon under static or cyclic stretching (10% strain, 0.33 Hz) during simultaneous exposure of GDF-5 for 48 h. They found that both the mechanical stretching and GDF-5 alone increased *COL I* and *SCX* expression, while additional synergistic effect of them was not observed [53]. It is possible that one of the stimulations was dominant and covered the effect of the other on inducing tenogenic differentiation [18]. In addition, the types and parameters of mechanical stimulation, as well as the concentration and delivery time of growth factors, may affect the performance of the synergistic effect on inducing tendon differentiation. During tendon repair or regeneration, growth factors may play a role at only certain times or phases and therefore dynamic release of growth factors or a stepwise-treated strategy may have better effects [18]. It requires further understanding of the mechanisms about how growth factors regulate tendon differentiation. In conclusion, the combination of different mechanical and biochemical stimulus is a potential strategy to superiorly construct an engineered tendon; however, the optimal set-up needs to be further explored.

7. Conclusion

Mechanical stimulation is an important regulatory factor in tendon tissue engineering, which can induce the

differentiation of stem cells into tenocytes and improve the performance of engineered tendon constructs. Dynamic uniaxial stretching simulates the biomechanical conditions of natural tendons by inducing tenogenic differentiation, alignment of cells and ECM, and promotes tendon repair and regeneration. The frequency of an effective protocol in bioreactors is usually not higher than 1 Hz, and between 1% and 12% strain with sufficient rest intervals. However, an optimal protocol is dependent on other conditions as well, such as cell types and loading systems. Natural mechanical stimulation *in vivo* facilitates the formation of a more mature tendon-like tissue in the engineered tendon construct. Although some progress has been made in studying the mechanisms of mechanical stimulation to activate tenogenic differentiation, this process is still not fully elucidated and needs further investigation. In addition, a future potential in the field of tendon tissue engineering could be the development of magnetic force, as well as combining mechanical and biochemical stimulation to obtain the synergistic effect to facilitate the development of the ideal engineered tendons.

Conflicts of Interest

The authors declare that they have no competing interests.

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