Stem Cells International

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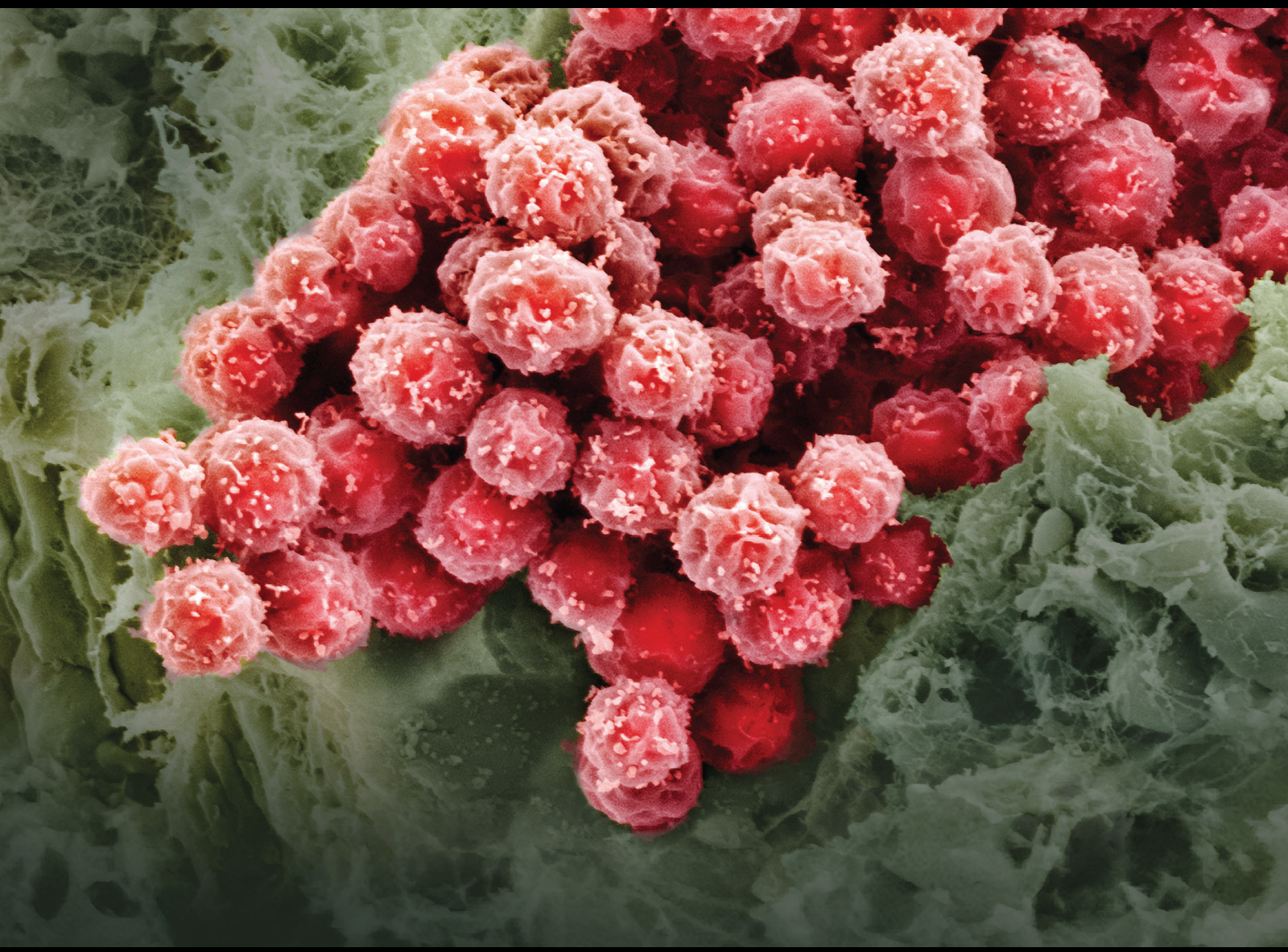
Lead Guest Editor: Bo Gao
Guest Editors: Ruoxian Deng and Beier Luo



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Contents

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Research Article

Aucubin Impeded Preosteoclast Fusion and Enhanced CD31^{hi} EMCN^{hi} Vessel Angiogenesis in Ovariectomized Mice

Ziyi Li,[1,2] **Chang Liu**,[1,2] **Xiaoli Liu**,[3] **Na Wang**,[1,2] **Liu Gao**,[1,2] **Xiaoxue Bao**,[1,2] **Sijing Liu**,[4] **and Peng Xue**[1,2]

[1]*Department of Endocrinology, The Third Hospital of Hebei Medical University, Shijiazhuang 050051, China*
[2]*Key Orthopaedic Biomechanics Laboratory of Hebei Province, Shijiazhuang 050051, China*
[3]*Department of Pediatric Dentistry, School and Hospital of Stomatology & Hebei Key Laboratory of Stomatology, Hebei Medical University, Shijiazhuang 050017, China*
[4]*Editorial Department of Hebei Medical University, Hebei Medical University, Shijiazhuang 050017, China*

Correspondence should be addressed to Sijing Liu; hbydxbl@126.com and Peng Xue; hebmuxuepeng@163.com

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Osteogenesis is tightly correlated with angiogenesis during the process of bone development, regeneration, and remodeling. In addition to providing nutrients and oxygen for bone tissue, blood vessels around bone tissue also secrete some factors to regulate bone formation. Type H vessels which were regulated by platelet-derived growth factor-BB (PDGF-BB) were confirmed to couple angiogenesis and osteogenesis. Recently, preosteoclasts have been identified as the most important source of PDGF-BB. Therefore, inhibiting osteoclast maturation, improving PDGF-BB secretion, stimulating type H angiogenesis, and subsequently accelerating bone regeneration may be potent treatments for bone loss disease. In the present study, aucubin, an iridoid glycoside extracted from *Aucuba japonica* and *Eucommia ulmoides*, was found to inhibit bone loss in ovariectomized mice. We further confirmed that aucubin could inhibit the fusion of tartrate-resistant acid phosphatase $(TRAP)^+$ preosteoclasts into mature osteoclasts and indirectly increasing angiogenesis of type H vessel. The underlying mechanism is the aucubin-induced inhibition of MAPK/NF-κB signaling, which increases the preosteoclast number and subsequently promotes angiogenesis via PDGF-BB. These results prompted that aucubin could be an antiosteoporosis drug candidate, which needs further research.

1. Introduction

The functional changes of osteoblasts and osteoclasts cause dynamic changes in the skeletal system. Uncoordinated action between osteoclasts and osteoblasts might result in many bone loss diseases, such as osteoporosis. Recently, our understanding of bone disease has expanded beyond the bone itself, and the relationship between the bone and bone microenvironment, especially blood vessels, has been gradually recognized. Type H vessels, a specific capillary subtype featuring CD31 and endomucin ($CD31^{hi}Emcn^{hi}$) markers, can provide nutrients and oxygen for bone tissue [1, 2]. Importantly, type H vessels are further identified around osteoprogenitors and interconnect the processes of bone formation and bone absorption during bone regeneration [3, 4]. The combination of angiocrine factors derived by type H vessels, osteoblasts, and osteoclasts could couple the interreaction of the bone microenvironment as well as the coordinating of angiogenesis and osteogenesis [5–9]. However, the relative abundance of type H vessels is low and reduces with the increasing of aging and the progressing of diseases [2].

A previous study showed that PDGF-BB produced by preosteoclasts had the ability to promote type H vessel angiogenesis and subsequently increase osteogenesis [10]. However, with the maturation of osteoclasts, the amount of PDGF-BB secreted by osteoclasts gradually decreased. Therefore, inhibiting the maturation of preosteoclasts can inhibit bone resorption and importantly promote bone formation through promoting angiogenesis of type H

vessels and might be promising therapeutic strategies for osteoporosis.

Aucubin is an iridoid glycoside extracted from *Aucuba japonica* and *Eucommia ulmoides* leaves. It has multiple functions, such as anti-inflammatory, antioxidative, cardioprotective, and neuroprotective effects [11–16]. In recent years, studies have found that aucubin is also related to bone metabolism by increasing bone formation [17, 18]. In addition, aucubin has been reported to promote angiogenesis in a mouse hindlimb ischaemia model [19]. Aucubin can play an antioxidant role by suppressing the NF-κB signaling pathway [11, 20], which is also a very important signal during the process of osteoclastogenesis. However, the effects of aucubin on osteoclasts and type H vessels angiogenesis remain unknown. In this research, we explored whether aucubin could inhibit the maturation of preosteoclasts and amplify angiogenesis by upregulating the secretion of PDGF-BB.

2. Materials and Methods

2.1. Animals and Treatments. All experiments of animal in this present research were under the supervision of the ethics committee of the Third Hospital of Hebei Medical University. The BALB/c female mice were purchased from the animal experimental center of Hebei Medical University and maintained until use. All mice were bred in a standard environment room with ad libitum access to water and food. All the animals were randomly assigned to the following three groups: (1) the sham group (sham-operated group +PBS), (2) the ovariectomy (OVX+PBS) group, and (3) the aucubin group (OVX+aucubin). Ovariectomy was performed by bilateral removal of ovaries at the 12 weeks old. One week after OVX operation, the aucubin group received 5 mg/kg aucubin by intraperitoneal injection every two days. Correspondingly, the OVX group was injected with the same amount of PBS at the same frequency. All mice were sacrificed one month later; blood and femurs were collected for further experiments.

2.2. Micro-CT Analysis. Micro-CT scans in isolated bones were performed using SkyScan1176 (Bruker, Belgium) μCT scanner. 9 μm per pixel resolution was set in the study. The scanning voltage is 65 kV, and the current is 153 μA. The trabecular parameters of femoral metaphysis were analyzed by data analysis software (CTAn, v1.9, SkyScan) and 3D model visualization software (CTVol, v2.0, SkyScan). The 3D analysis of trabecular bone was performed by creating cross-sectional images of femur. The trabecular parameters were measured by trabecular bone volume/total volume (BV/TV, %), trabecular number (Tb.N, mm^{-1}), trabecular separation (Tb.Sp, μm), and trabecular thickness (Tb.Th, mm), and the differences among the groups were compared.

2.3. Haematoxylin and Eosin (HE) Staining and Tartrate-Resistant Acid Phosphatase (TRAP) Staining. HE staining and TRAP staining were performed on 4 μm sections of the femur after 3 weeks of bone decalcification in 10% EDTA solution. For HE staining, the 4 μm thick section samples were processed for staining after fixation, decalcification and paraffin embedding. The images were obtained by an optical microscope, and the relevant bone histological parameters were calculated. For TRAP staining, a TRAP staining kit (Sigma-Aldrich) was used after the tissue was fixed and embedded according to the instructions. After trap staining, samples were analyzed by optical microscope. TRAP-positive cells with more than three nuclei were defined as osteoclasts, and TRAP-positive cells with less than three nuclei were preosteoclasts.

2.4. Immunofluorescent Analyses of Sample Sections. For immunofluorescent (IF) staining, the femur samples were cut into 16 μm thick sections after being fixed for 24 hours, decalcified for 3 weeks, and embedded in 8% gelatin. Briefly, the 16 μm thick sections were first washed with 1% PBST (1% Triton X-100 dissolve in PBS) for 30 min three times before the slices were incubated with 5% BSA at 37°C for 1 h. Then, the samples were incubated with primary antibodies against CD31 (Abcam, MA, USA), EMCN (Abcam, MA, USA) and osteocalcin (Abcam, MA, USA) overnight at 4°C. Finally, the corresponding fluorescence-conjugated secondary antibodies were stained at 37°C for 1 h.

2.5. Cell Culture. RAW264.7 cells, the macrophage cell line, was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). α-MEM supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 1% penicillin-streptomycin was used to culture the RAW264.7 cells. To induce osteoclast differentiation, the cells were treated with 50 ng/mL receptor activator for nuclear factor κB ligand (RANKL) with different concentrations of aucubin (0, 1, or 5 μM) for 6 days. Microvascular endothelial cells of mice (MMECs) were purchased from Procell (Wuhan, China) and cultured in endothelial cell culture medium (ECM). All cells were cultured at 37°C with 5% CO_2.

2.6. Cell Viability Assay. Cell viability was performed by a cell Counting Kit-8 (CCK-8) assay (Zomanbio, Beijing, China) according to the manufacturer's instructions. First, RAW264.7 cells were seeded onto 96-well plates at a density of 5×10^3 cells per well. After treatment with aucubin (0, 1, or 5 μM) for 24, 48, and 72 hours, 10 μL CCK-8 solution was added. Finally, the absorbance at 450 nm was measured by a microplate spectrophotometer (BioTek Instruments, San Jose, CA, USA) after being incubated at 37°C for another 4 h.

2.7. TRAP Staining of Cells. The macrophage cell lines were fixed with 4% paraformaldehyde after several days of osteoclastogenesis induction. Then, TRAP staining was performed using a TRAP staining kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturers' instructions. After trap staining, samples were analyzed by optical microscope. Same as tissue samples, TRAP+ mononuclear cells and multinucleated cells with more than three nuclei were identified as preosteoclasts and osteoclasts, respectively.

2.8. Immunofluorescent Analyses of Cells. For immunofluorescent staining, cells were first treated in Triton X-100 for

half an hour before blocking with 10% BSA. Then, the cells were stained with primary antibodies against NFATc1 (Santa Cruz, CA, USA) or p65 (CST, MA, USA) at 4°C overnight. Finally, the samples were stained with secondary antibodies at room temperature for 1 h.

2.9. Actin Ring-Formation Assay. Briefly, the macrophage line cells were first stimulated with Rankl and varying doses of aucubin for 6 days. Then, the cultured osteoclasts were permeabilization with 0.5% Triton X-100 after being fixed with 4% paraformaldehyde. Subsequently, FITC-conjugated phalloidin was used to stain F-actin rings, and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) dye. Finally, a scanning confocal microscopy (Nikon, Tokyo, Japan) was used to take fluorescence images. ImageJ software was used to analysis the number and size of F-actin rings.

2.10. Migration Assay. In the migration assay, MMECs were seeded on 6-well culture plates and cultured until the single cell layer was confluent. Then, cells were cultured in different treatments. MMECs were used in two parallel experiments. In the first experiment, MMECs were cultured in endothelial cell culture medium with or without aucubin. In the second experiment, MMECs were cultured in different conditioned medium (CM). According to the treatments, the groups were divided into the following groups: the vehicle (CM was harvested from RAW264.7 cells)+IgG (Abcam, Cambs, Britain) group, the Rankl (CM was gathered from RAW264.7 cells stimulated with Rankl)+IgG group, the Rankl+Aucubin (CM was gathered from RAW264.7 cells which were stimulated with Rankl and Aucubin at the same time)+IgG group, and the Rankl +Aucubin (CM collected from RAW264.7 cells stimulated with RANKL and aucubin)+PDGF-BB antibody (R&D, Minneapolis, USA) group. The cell monolayer was scratched with the tip of a pipette gun followed by washing with PBS. After 0, 24, and 48 hours, the wounds were acquired by microscopy and measured by ImageJ software.

2.11. Tube Formation Assay. To perform tube formation assays, 50 μL/well of Matrigel (BD, USA) was spread on 96-well culture plates and incubated at cell incubator for half an hour. Then, MMECs were seeded on solidified gel in the 96-well plate and cultured under different treatments with a density of 1×10^4 cells/well. MMECs were used in two parallel experiments similar to the scratch test. First, MMECs were cultured in endothelial cell culture medium with or without aucubin. Subsequently, MMEC groups were divided into the following groups: the vehicle (CM was harvested from RAW264.7 cells)+IgG group, the Rankl (CM was gathered from RAW264.7 cells stimulated with Rankl)+IgG group, the Rankl+Aucubin (CM was gathered from RAW264.7 cells which were stimulated with Rankl and Aucubin at the same time)+IgG group, and the Rankl +Aucubin (CM collected from RAW264.7 cells stimulated with RANKL and aucubin)+PDGF-BB antibody group. After 6 hours, tube formation was acquired by microscopy and measured by Image-Pro Plus 6 software.

2.12. Enzyme-Linked Immunosorbent Assay (ELISA). The concentrations of CTX-1, OCN, PDGF-BB, and VEGF in the blood serum, bone tissue, or conditioned medium were measured using commercial ELISA Kits according to the manufacturers' instructions. The content of each group was detected by a microplate reader. PDGF-BB ELISA Kit was obtained from Cusabio (Wuhan, China). VEGF, CTX-1, and OCN ELISA Kit were purchased from Multi Sciences LTD (Hangzhou, China).

2.13. Real-Time RT-PCR. TRIzol reagent (Tiangen, Beijing, China), RevertAid™ First Strand cDNA Synthesis Kit (Thermo, Waltham, USA), and SuperReal PreMix Plus (Tiangen, Beijing, China) PCR Kit were used to perform the real-time RT-PCR analysis. First, total RNA was extracted by TRIzol reagent according to the protocols before reverse-transcribed into cDNA. Then, real-time RT-PCR was performed following the instructions. During the process, GAPDH was selected as the internal control, and the $2^{-\Delta\Delta Ct}$ method was used to evaluate relative gene expression. The specific primer sequences used for the experiments are as follows: GAPDH: 5′-AGTTCAACGGCACAGTCAAGG-3′, 5′-AGCACCAGCATCACCCCAT-3′; Atp6v0d2: 3′-AGCAAAGAAGACAGGGAG-5′, 5′-CAGCGTCAAACAAAGG-3′; NFATc1: 3′-CAACGCCCTGACCACCGATAG-5′, 5′-GGCTGCCTTCCGTCTCATAGT-3′; cathepsin K: 3′-CAGCAGAACGGAGGCATTGA-5′, 5′-CTTTGCCGTGGCGTTATACATACA-3′; PDGF-BB: 3′-CCTCGGCCTGTGACTAGAAG-5′, 5′-CCTTGTCATGGGTGTGCTTA-3′; DC-STAMP: 3′-GATCACCTGTGTTTTCCTATGC-5′, 5′-CAATCAAAGCGTTCCTACCTTC-3′; C-fos: 3′-CGGGTTTCAACGCCGACTA-5′, 5′-TTGGCACTAGAGACGGACAGA-3′; MMP-9: 3′-GCGTCGTGATCCCCACTTAC-5′, 5′-CAGGCCGAATAGGAGCGTC-3′; VEGF: 3′-GAGGTCAAGGCTTTTGAAGGC-5′, 5′-CTGTCCTGGTATTGAGGGTGG-3′; MMP-2: 3′-TCAAGTTCCCCGGCGATG-5′, 5′-AGTTGGCCACATCTGGGTTG-3′.

2.14. Western Blot Analysis. RIPA lysis buffer (Beyotime, Shanghai, China), 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) were used to perform the Western blot analysis. Briefly, RIPA lysis buffer was used to obtain total proteins. Then, 25 μg of protein was resolved by SDS-PAGE and subsequently transferred to PVDF membranes. Ten percent BSA in Tris-buffered saline mixed with Tween 20 (TBST) was used to block the PVDF membranes. The membranes were stained with primary antibodies against GAPDH (1 : 10000), PDGF-BB (1 : 1000), cathepsin K (1 : 1000), c-fos (1 : 1000), NFATc1 (1 : 1000), p-JNK (1 : 500), JNK (1 : 1000), p-p38 (1 : 1000), p38 (1 : 1000), p-p65 (1 : 1000), p65 (1 : 1000), p-IKBα (1 : 1000), or IKBα (1 : 1000) at 4°C for 8 h. Finally, the membranes were incubated with secondary antibodies (1 : 20,000, Rockland, USA) for 1 hour at room temperature. An Odyssey infrared imaging system was used to detect the integrated intensity for each group. Antibodies of NFATc1,

PDGF-BB, and Emcn were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p-JNK, JNK, p-p38, p38, p-IKBα, IKBα, p-p65, and p65 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). c-Fos, CD31, OCN, and GAPDH antibodies were purchased from Abcam (Cambridge, MA, USA).

2.15. Statistical Analysis. The results in the present study are expressed as the mean ± standard deviation. The outcomes were from at least 3 independent replications. The Kolmogorov-Smirnov test was used to test the normality assumptions of experimental data. An independent samples t-test was used for the comparison between two groups, and one-way analysis of variance (ANOVA) followed by the Student Newman Keuls (S-N-K) post hoc analysis was used for the analysis among multiple groups. For the nonparametric data, the Kruskal-Wallis test was performed. All the data analysis was using SPSS 20.0 software, and statistical significance was measured by $P < 0.05$.

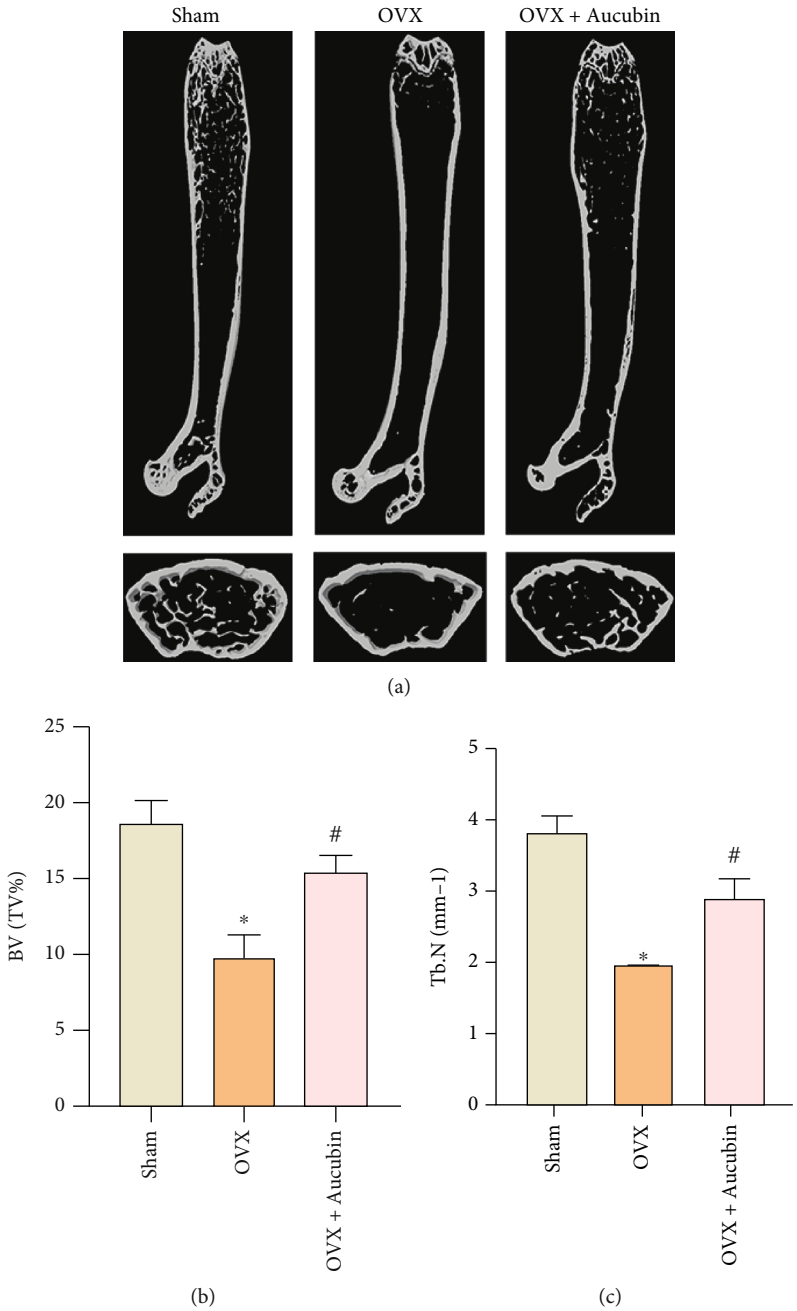
3. Results

3.1. Aucubin Inhibits OVX-Induced Bone Loss and Augments Type H Vessel Formation. In order to assess the effect of aucubin on bone loss and type H formation, an OVX mouse model was generated in the present study. OVX clearly impaired the bone structure compared to the sham group, while intraperitoneal injection of aucubin in OVX mice partly attenuated this bone loss as shown by micro-CT scans (Figure 1(a)). Correspondingly, quantitative analyses of the Tb.N, BV/BT%, and Tb.Th were significantly decreased after OVX surgery; however, these reductions were mitigated by the aucubin treatment (Figures 1(b)–1(d)). Aucubin also reduced the increased trabecular bone volume fraction (Tb.Sp) caused by OVX (Figure 1(e)). HE staining also verified that aucubin could rescue OVX-induced bone loss (Figures 1(f) and 1(g)). Consistent with this finding, the ELISA results indicated that aucubin significantly abolished the decrease in serum levels of OCN as well as the increase of CTX-1 content in serum induced by OVX (Figures 1(h) and 1(i)).

TRAP staining was used to confirm the inhibitory function of aucubin on osteoclastogenesis in vivo. Consistent with the change in the bone resorption index in the serum, OVX mice had a larger number of multinucleated osteoclasts. However, aucubin administration significantly reduced the number of mature osteoclasts as well as increased the number of preosteoclasts (Figures 2(a) and 2(b)). The results confirmed that aucubin has an inhibitory role in osteoclast maturation. As the number of osteoclasts in each group was different, the content of PDGF-BB in bone marrow changed between groups. PDGF-BB decreased after the OVX operation. However, the PDGF-BB content increased after the aucubin intervention (Figure 2(c)). Emcn and CD31 double IF staining were used to confirm the effects of aucubin on angiogenesis of type H blood vessel. The quantity of type H vessels was decreased by OVX, and aucubin treatment results in a remarkable upregulation in this particular type vessel (Figures 2(d) and 2(e)). In addition, the influence of aucubin on bone formation was also detected. Aucubin partially alleviated the decrease in OCN in the bone surface caused by OVX (Figures 2(f) and 2(g)). The in vivo experiments suggested that aucubin treatment decreases the number of osteoclasts, promotes the angiogenesis of type H vessels, and increases bone formation. All animal experiments indicated that the preosteoclast-induced angiogenesis facilitation might be a potential mechanism by which aucubin protects skeleton.

3.2. Aucubin Increases the Preosteoclast Number and Inhibits Osteoclast Maturation. CCK-8 assay was performed to detect the effect of aucubin on cell viability. The results were detected at 1, 2, and 3 days after administration of aucubin. There were no significant influences of aucubin on cell viability of RAW264.7 cells at low concentrations (1 μM) or high concentrations (5 μM) (Figure 3(a)). In order to detect the influence of aucubin on osteoclastogenesis, TRAP staining was performed 6 days after the RAW264.7 cells were stimulated by Rankl and administrated with or without aucubin. The results showed that the number of mature osteoclasts in RAW264.7 cells increased under Rankl induction while the fusion of TRAP+preosteoclasts was inhibited by the aucubin treatment, with the inhibitory effect more obvious with a high dose of aucubin (Figures 3(f) and 3(g)). These findings indicated that aucubin had the ability to prevent the maturation process of preosteoclasts into multinucleated osteoclasts. RT-PCR analysis was used to evaluate the expression of osteoclast-related mRNA and fusion-related mRNA, including NFATc1, cathepsin K (CTSK), ATP6V0D2, and DC-STAMP. As shown in Figures 3(b)–3(e), aucubin significantly suppressed osteoclast-related mRNA (NFATc1 and CTSK) as well as osteoclast fusion mRNA stimulated by RANKL in macrophage cells. In parallel tests, Western blot results indicated that c-Fos and NFATc1 protein were downregulated by the addition of aucubin (Figures 3(h)–3(j)). Next, IF staining was performed to assess the effect of aucubin on NFATc1, a key factor in osteoclast differentiation. The results showed the increase in NFATc1 induced by RANKL after 12 hours was partially offset by aucubin (Figures 3(k) and 3(l)). These parts of results indicate that aucubin depresses the fusion of preosteoclasts by inhibiting osteoclast-related factors.

3.3. Aucubin Promotes Angiogenesis via Increasing the Production of PDGF-BB In Vitro. IF, ELISA, and Western blotting were performed to determine the influence of aucubin intervention on the production of PDGF-BB during the osteoclastic differentiation (Figures 4(a)–4(e)). The content of PDGF-BB increased by Rankl stimulation. Meanwhile, PDGF-BB further augmented with the inhibition of osteoclast precursor cell fusion by the aucubin treatment (Figures 4(a) and 4(b)). As confirmed by ELISA, Rankl increased the content of PDGF-BB in the supernatant and aucubin potentiated Rankl-induced PDGF-BB in a dose-dependent manner (Figure 4(c)). Consistent with the ELISA results, aucubin also upregulated the expression of PDGF-BB in protein and mRNA level (Figures 4(d)–4(f)). Thus,



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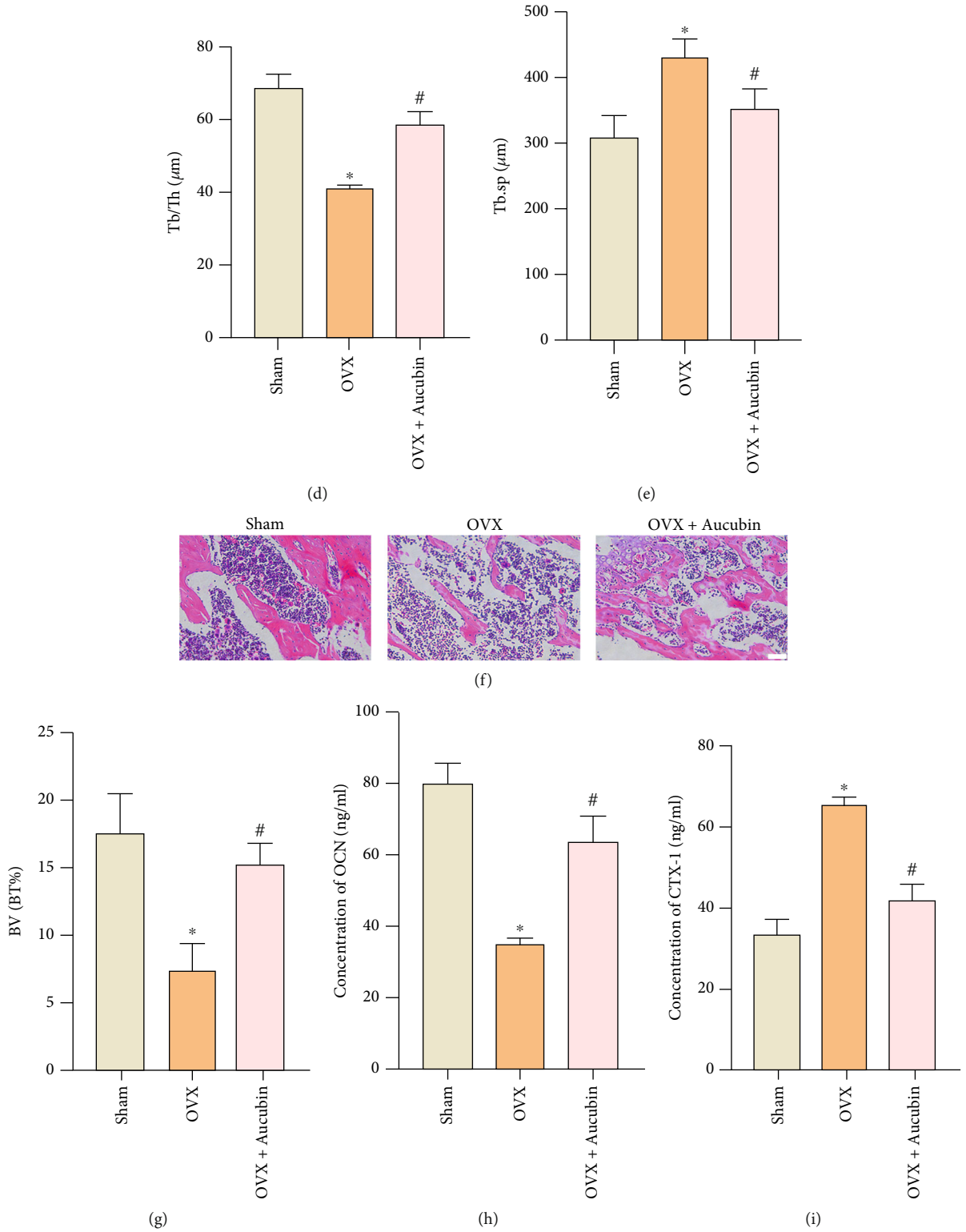


Figure 1: Aucubin ameliorated bone loss induced by OVX. (a) Micro-CT images and quantitative analysis. (b–e) of the mouse femurs from different groups. BV/TV: bone volume/total volume; Tb.N: trabecular number; Tb.Sp: trabecular separation; Tb.Th: trabecular thickness. (f) Representative haematoxylin and eosin (HE) staining of femurs; scale bar: 100 μm. (g) Quantification of BV/TV% of HE staining. (h, i) The concentrations of OCN and CTX-1 in the serum were detected by ELISA. $n = 5$ per group. $^{*}P < 0.05$ compared to the sham group. $^{\#}P < 0.05$ compared to the OVX group.

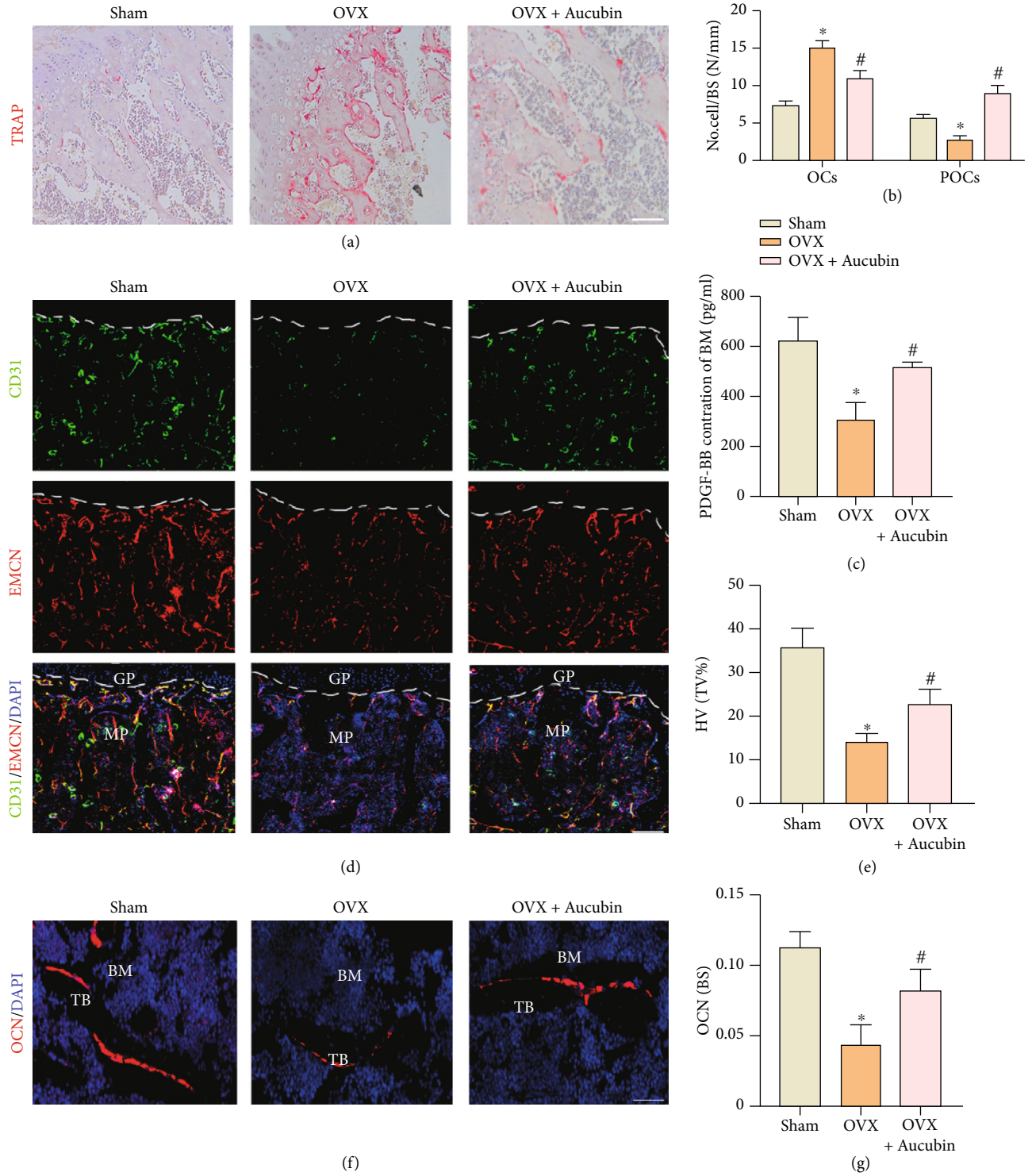
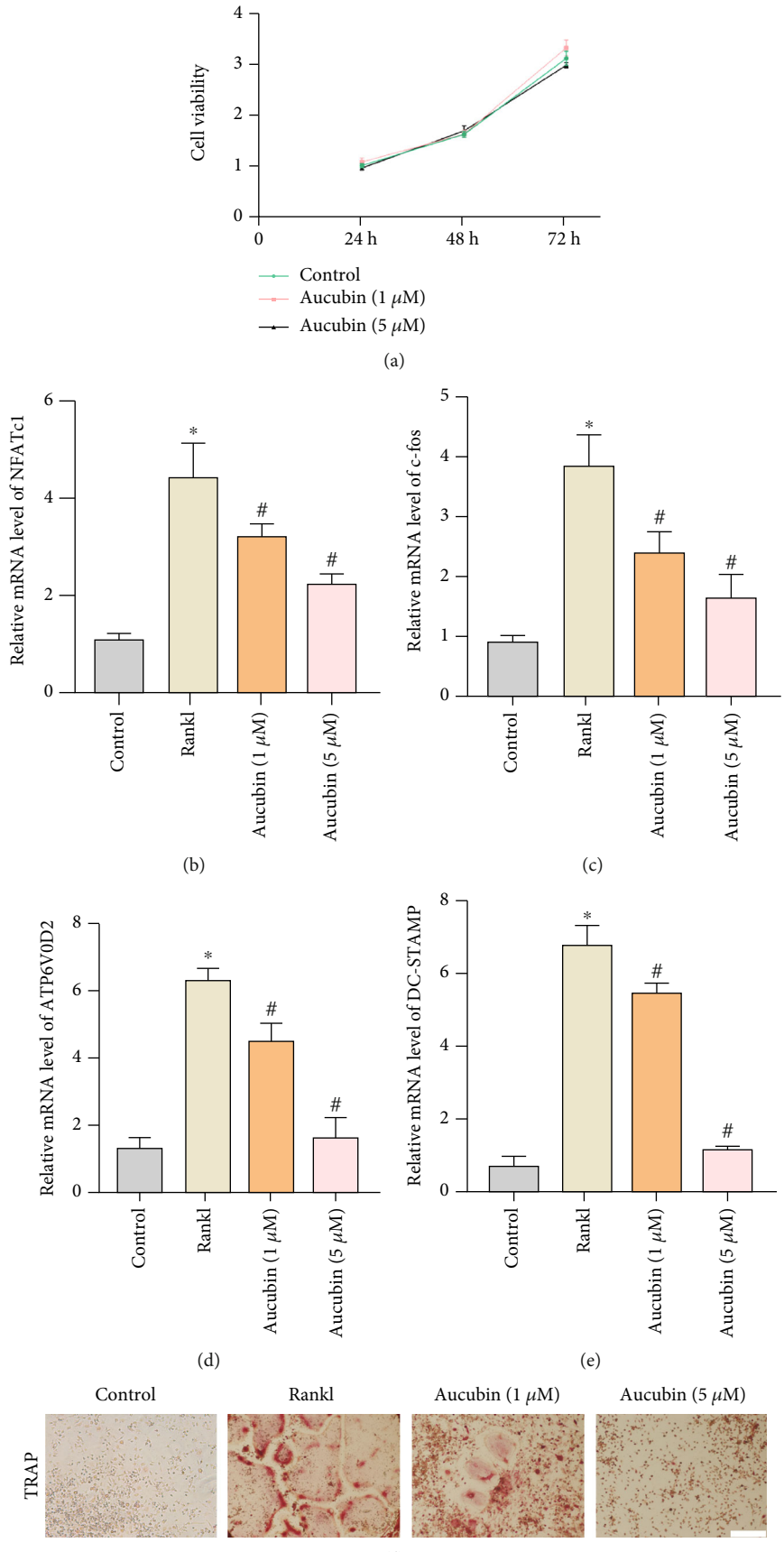


Figure 2: Aucubin represses osteoclast formation, promotes PDGF-BB secretion, and promotes type H vessel formation in OVX mice. (a) TRAP staining of the femora to calculate the numbers of osteoclasts and preosteoclasts of bone surface. Scale bar: 200 μm. (b) Quantification of OCs and POCs per bone surface of different groups. BS: bone surface. (c) Quantification of PDGF-BB levels in bone marrow was detected by ELISA and analyzed. (d) Type H vessels were detected by $CD31^{hi}Emcn^{hi}$double IF. Scale bar: 100 μm. (e) Type H vessels were quantitative analysis in each group. BM: bone marrow; GP: growth plate. $n = 5$ per group. (f) Representative images of IF staining of OCN, Scale bar: 50 μm. (g) The quantification of OCN+cell surfaces/bone surfaces. TB: trabecular bone; BM: bone marrow. $^{*}P < 0.05$ compared to the sham group; $^{\#}P < 0.05$ compared to the OVX group.



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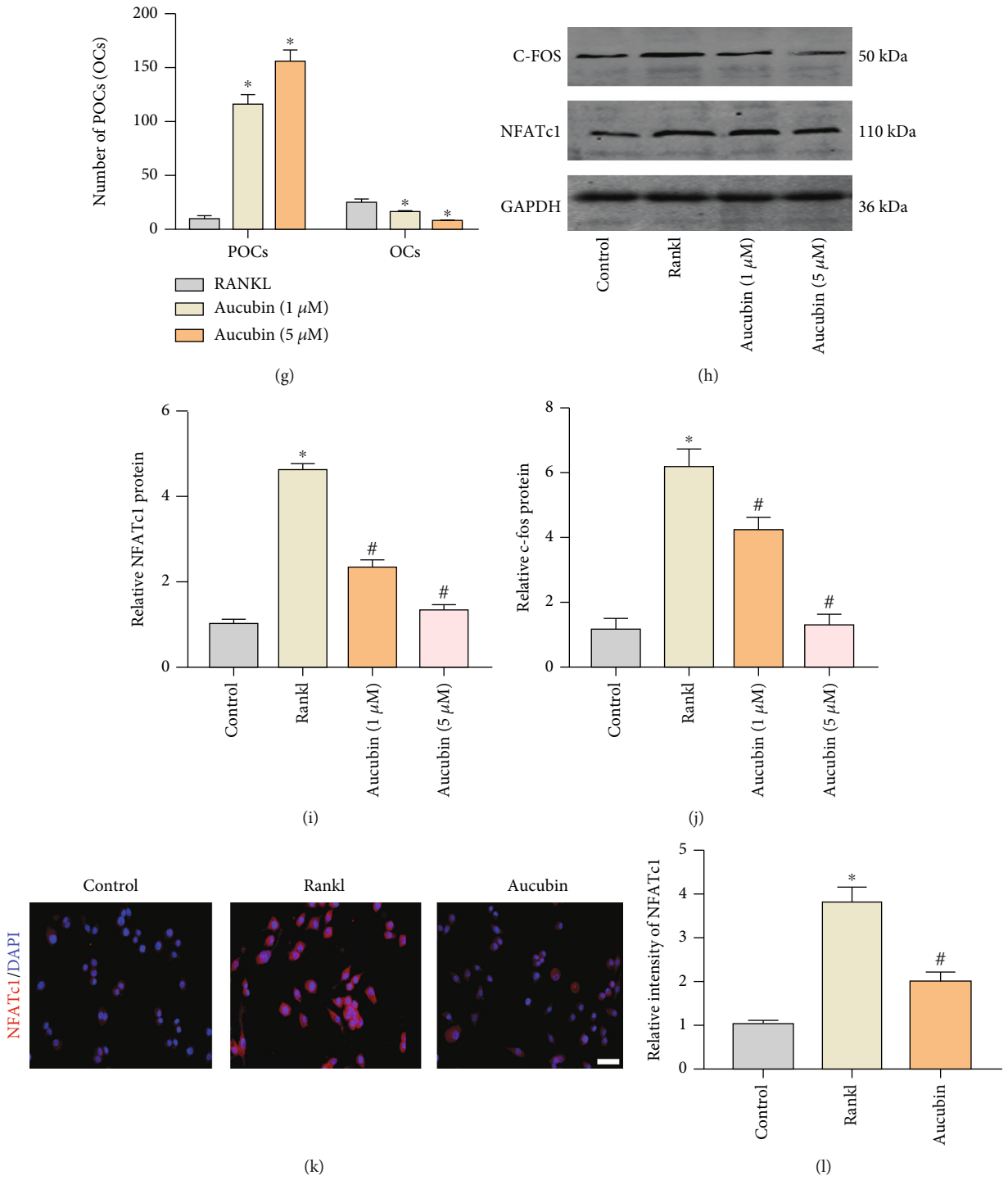
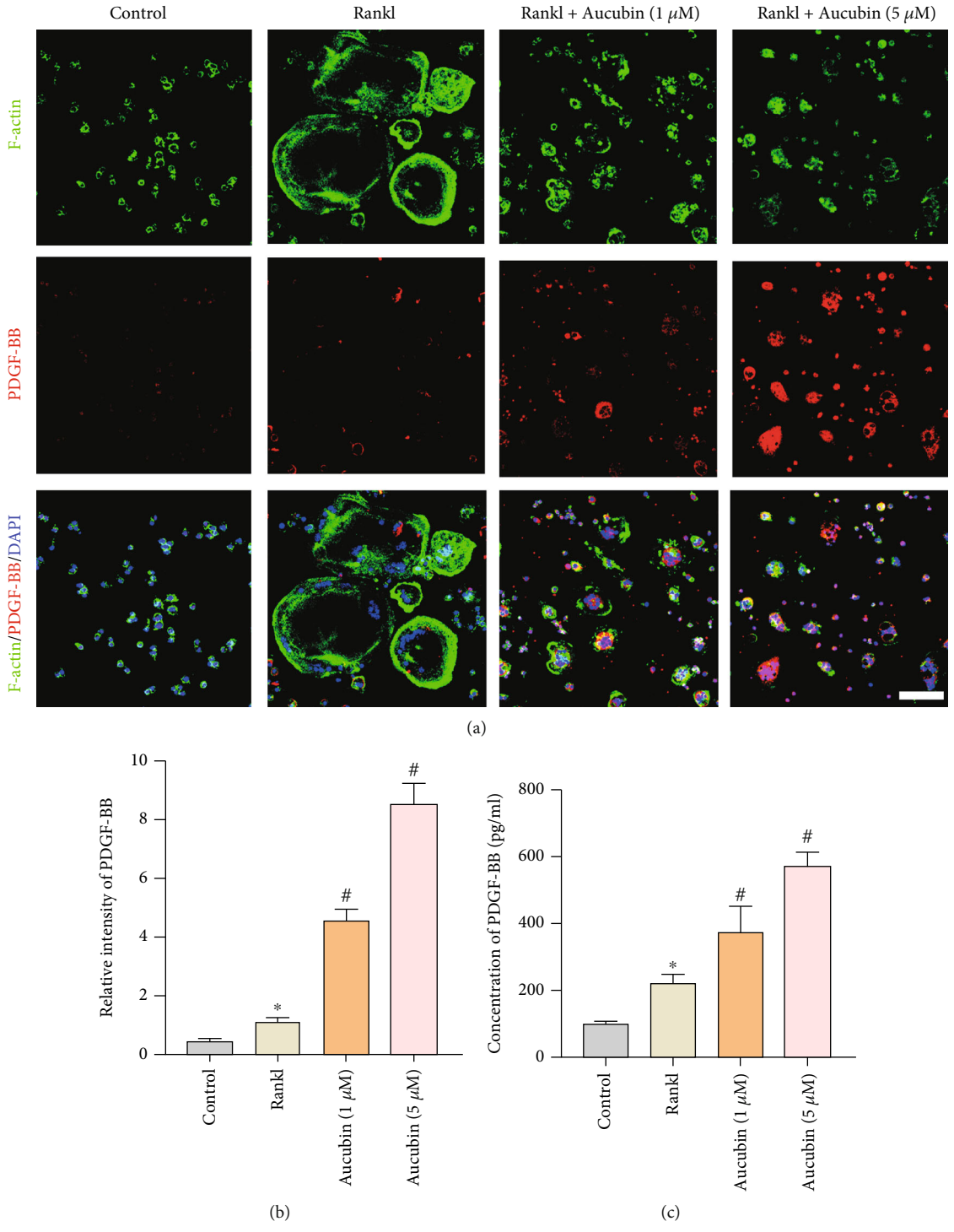


Figure 3: Aucubin increases the number of preosteoclasts in vitro. (a) Cell viability of RAW264.7 cells cultured with different concentrations of aucubin (0, 1 μM, and 5 μM) was analyzed via a CCK-8 assay. (b–e) Relative mRNA expression levels of NFATc1, c-FOS, DC-STAMP, and ATP6V0D2 were analyzed. (f) Representative images of TRAP staining on day 6. Scale bar: 200 μm. (g) Quantification of osteoclasts (OCs) and preosteoclasts (POCs) on day 6. (h–j) Protein levels of c-Fos and NFATc1 were analyzed by WBs. (k) IF staining was performed to observe the location of NFATc1. Scale bar: 50 μm. (l) The quantification of the relative NFATc1 fluorescence intensity in different groups. $^{*}P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the Rankl group.

we suggested that aucubin promoted the secretion of PDGF-BB through raising the number of preosteoclasts.

To assess whether aucubin had the direct effect on angiogenesis, the scratch wound assay (Figures 5(a) and 5(b)) and tube formation assay (Figures 5(c) and 5(d)) were performed. The quantitative measurements revealed that aucubin had no significant effect on the migration ability and tube formation ability of MMECs. In order to confirm the



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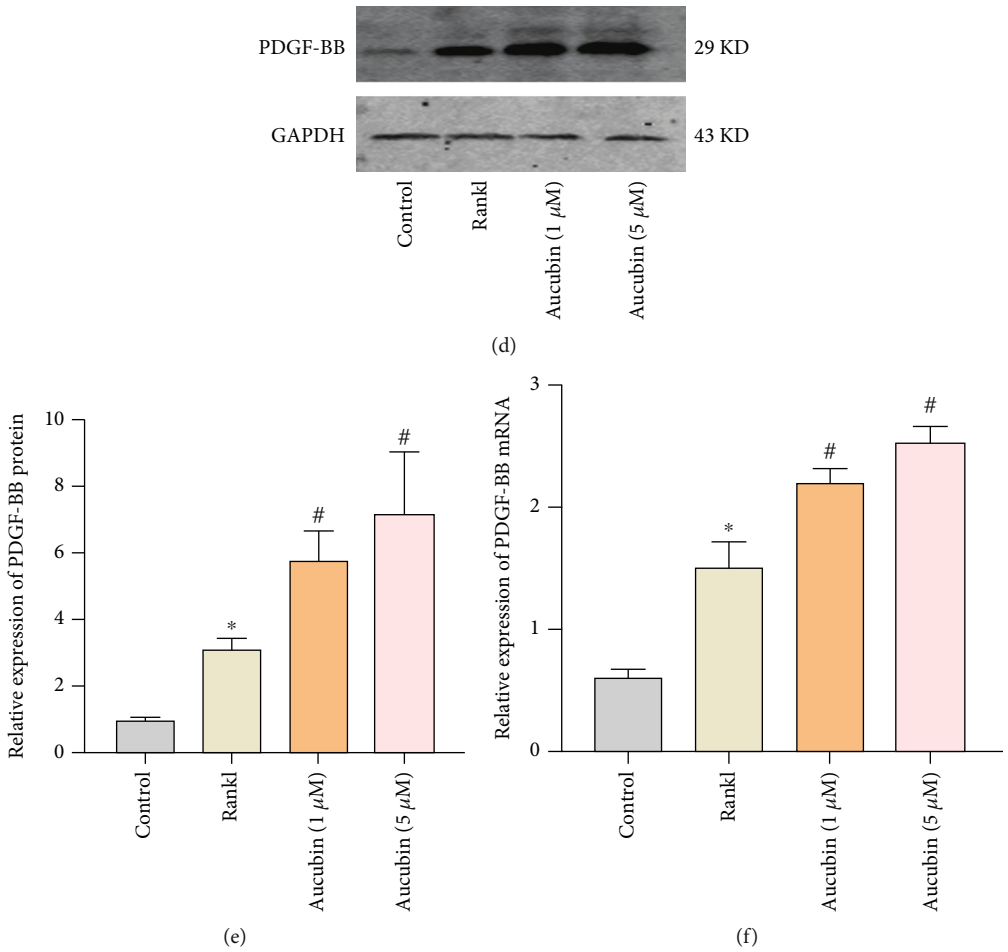
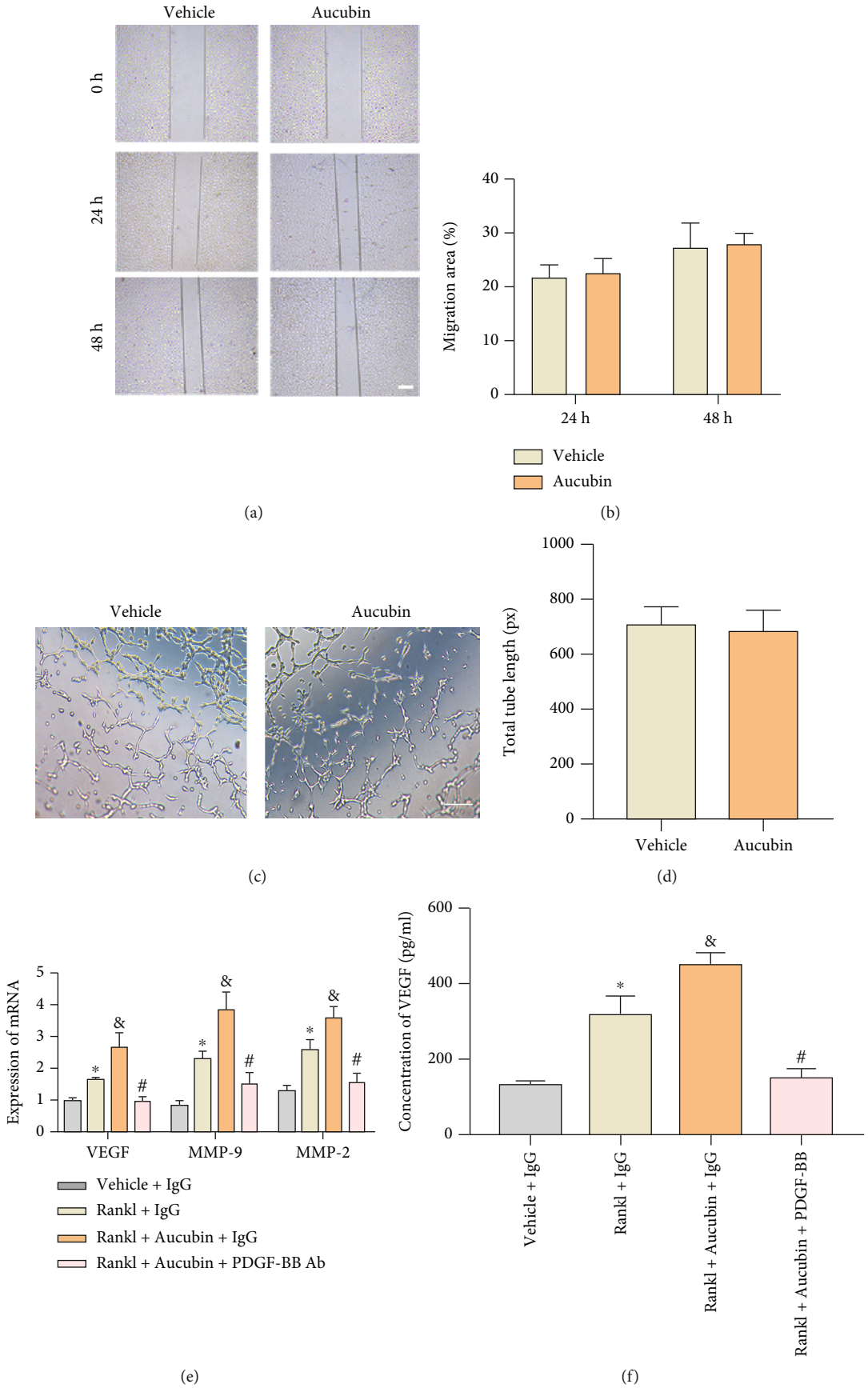


Figure 4: Aucubin augments the production of PDGF-BB in vitro. (a) IF staining was performed to observe the production of PDGF-BB. Scale bar: 50 μm. (b) Quantification of the relative PDGF-BB fluorescence intensity. (c) Concentration of PDGF-BB in CM was analyzed by ELISA. (d) Protein levels of PDGF-BB were analyzed by WBs. (e) Quantification of PDGF-BB protein expression with different treatments. (f) Quantification of PDGF-BB mRNA expression with different treatments. $^{*}P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the Rankl group.

effect of aucubin in preosteoclast-stimulated angiogenesis and migration, MMECs were cultured in different groups: the vehicle+IgG group, the Rankl+IgG group, the Rankl+Aucubin+IgG grpup, and Rankl+Aucubin+PDGF-BB antibody group. As shown in the scratch test, the migration ability of MMECs was significantly enhanced in the Rankl+IgG group compared to the vehicle+IgG group, and the supernatant of the aucubin+Rankl group enhanced the effect when combined with that of the Rankl group (Figures 5(g) and 5(h)). To further verify the changes mediated by PDGF-BB, the PDGF-BB-neutralizing antibody was further added to the RANKL+aucubin group, and the cell migration was inhibited. The results of the tube formation experiment are similar to those of the scratch test. The CM of Aucubin further expanded the total tube length compared to the Rankl+IgG group. However, this increase was attenuated by PDGF-BB-neutralizing antibodies (Figures 5(i) and 5(j)). As evidenced by PCR, the expression of MMP-9, MMP-2, and VEGF mRNA of the aucubin+Rankl+IgG group was increased when compared to that of the Rankl+IgG group and PDGF-BB-neutralizing antibodies eliminated the increase of MMP-9, MMP-2, and VEGF mRNA caused by aucubin CM (Figure 5(e)). Consistent with the PCR results, the increased concentration of VEGF in the aucubin +Rankl+IgG group was blocked by neutralizing antibodies against PDGF-BB (Figure 5(f)). In summary, aucubin promotes angiogenesis via increasing the production of PDGF-BB of preosteoclasts.

3.4. Aucubin Inhibits RANKL-Induced MAPK and NF-κB Signaling. In order to elucidate the mechanism of aucubin on osteoclast formation and PDGF-BB production, the MAPK and NF-κB signaling pathways, which play important roles in osteoclastogenesis, were detected. Western blots confirmed that aucubin significantly suppressed the MAPK signaling pathway. The active forms of ERK, p38 and JNK at 20 and 30 minutes was inhibited by aucubin treatment compared with that of the Rankl group (Figures 6(a)–6(d)). To further clarify whether NF-κB signaling took part in the mechanisms underlying the effect of aucubin, IKBα and p65 protein were checked (Figures 6(e) and 6(f)). The outcomes revealed that aucubin suppressed the activation



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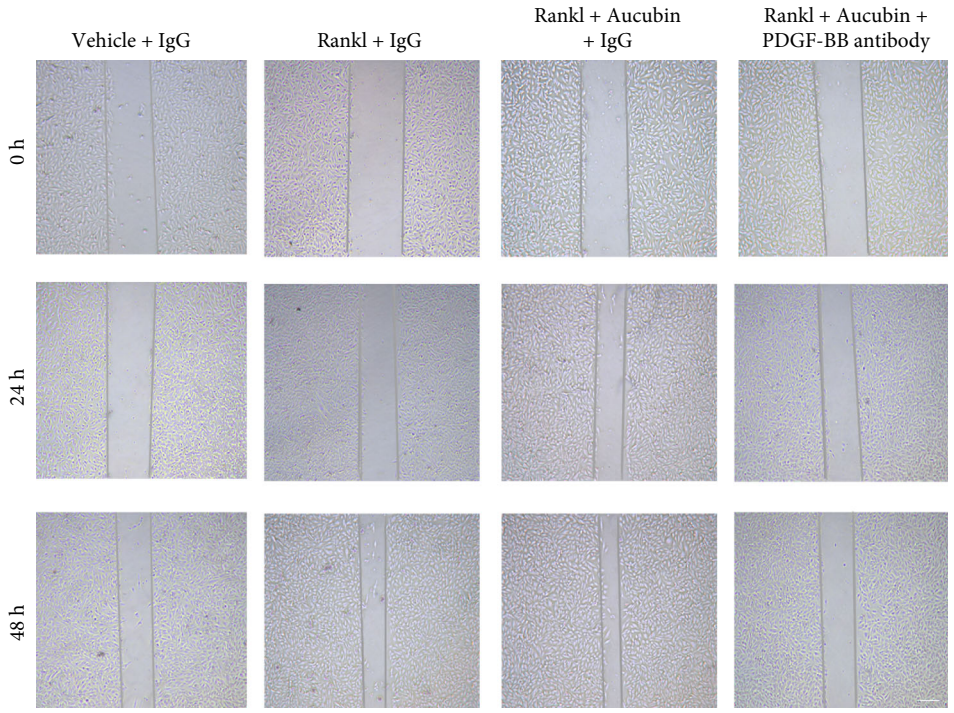
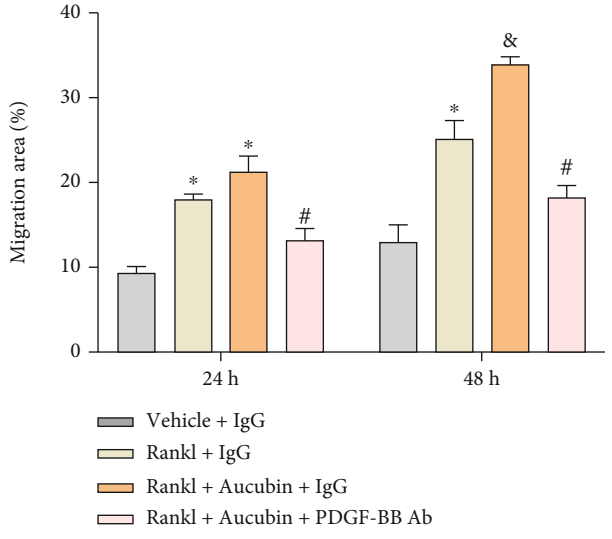


(g)



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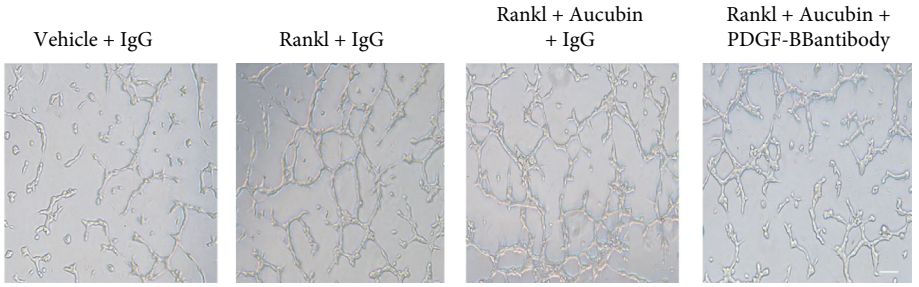

(i)

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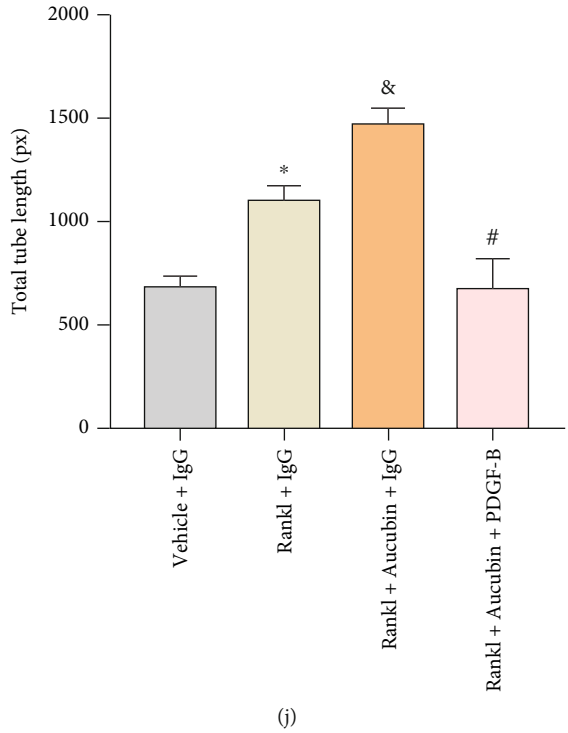


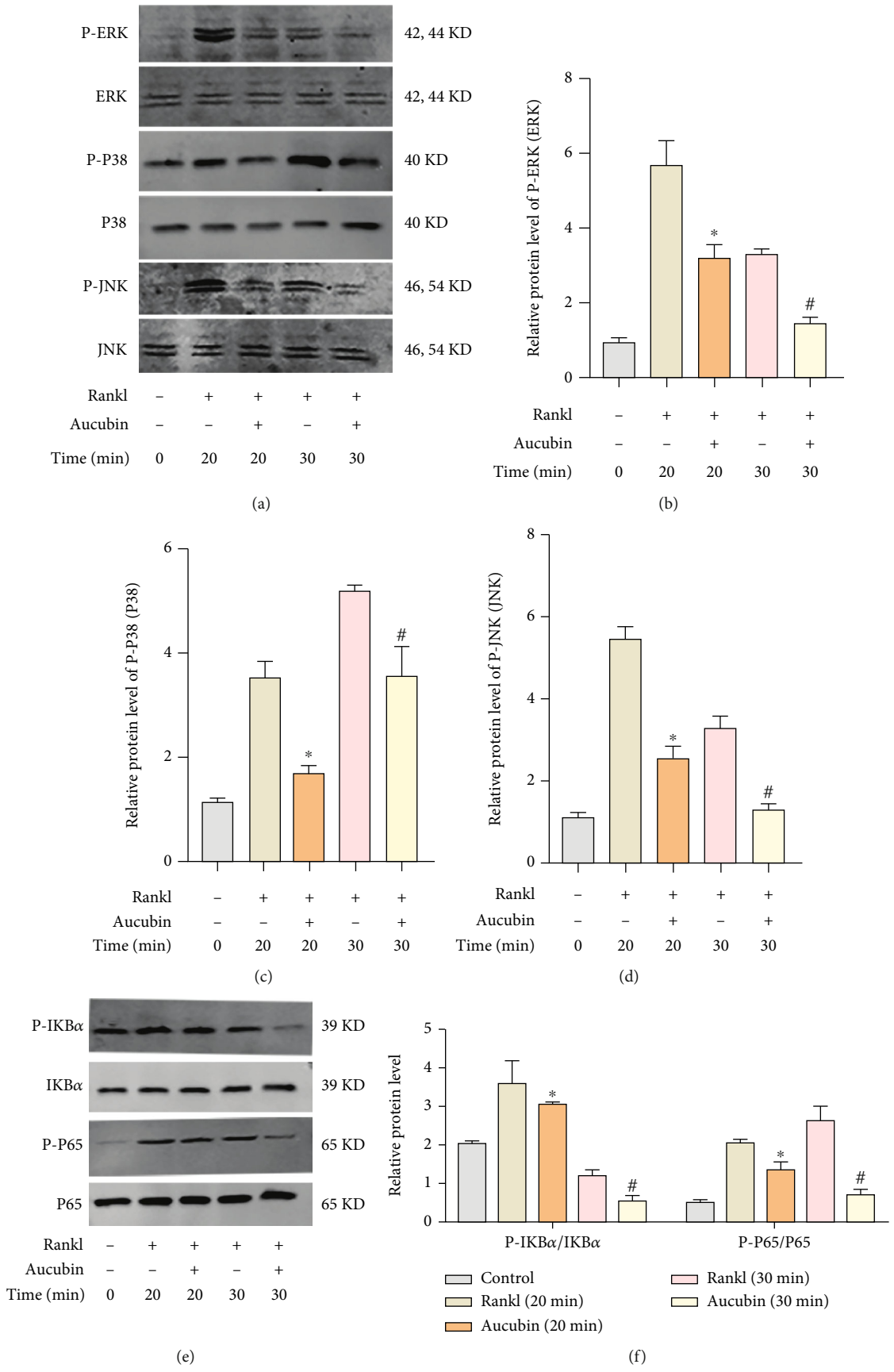
Figure 5: Aucubin promotes the proangiogenic effects of preosteoclasts on MMECs. (a) Representative images of the migration area of MMECs in different treatments. Scale bar: 200 μm. (b) The quantification of the migration area of MMECs in different treatments. Scale bar: 200 μm. (c) Representative images of tube formation in MMECs stimulated with different treatments. Scale bar: 200 μm. (d) The quantification of tube formation in MMECs stimulated with different treatments. (e) Relative mRNA expression levels of VEGF, MMP-9, and MMP-2 in MMECs with different CMs. (f) Concentration of VEGF in CM was analyzed by ELISA. (g) Representative images of the migration area of MMECs in different treatments. Scale bar: 200 μm. (h) The quantification of the migration area of MMECs in different treatments. $^{*}P < 0.05$ compared to the vehicle+IgG group; $^{\&}P < 0.05$ compared to the Rankl+IgG group; $^{\#}P < 0.05$ compared to the Rankl+Aucubin+IgG group. (i) Representative images of tube formation in MMECs stimulated with different treatments. Scale bar: 200 μm. (h) The quantification of tube formation in MMECs stimulated with different treatments. $^{*}P < 0.05$ compared to the vehicle+IgG group; $^{\&}P < 0.05$ compared to the Rankl+IgG group; $^{\#}P < 0.05$ compared to the Rankl+Aucubin+IgG group.

of IKBα (Figures 6(e) and 6(f)). IKBα regulates the activity of p65 in the NF-κB signaling pathway. In addition, the phosphorylation of p65 was also inhibited by aucubin (Figures 6(e) and 6(f)). Results of IF staining and qRT-PCR further showed that NF-κB translocation to the nucleus was activated by Rankl and inhibited by aucubin (Figures 6(g) and 6(i)). The above results indicated that aucubin inhibited Rankl-induced osteoclastogenesis through the MAPK and NF-κB signaling pathways.

4. Discussion

Osteoporosis, characterized by quantitative and qualitative deterioration of bone, has become one of the most serious chronic diseases [21]. The imbalance of bone formation and bone resorption is the most direct cause of osteoporosis. Recent studies have confirmed that bone microenvironment, especially the vascular system, plays a crucial part in maintaining the normal progression of bone metabolism [22]. Type H vessels that are mostly regulated by PDGF-BB are vital to bone remodeling [4]. Moreover, previous studies have affirmed that the suppression of osteoclasts differentiation at the precursor stage could promote the production of PDGF-BB [23–25]. Thus, blocking bone absorption and enhancing osteogenesis by promoting type H vessels might represent a new direction for the treatment of bone-lost diseases. In this research, it is demonstrated that aucubin inhibited the preosteoclast fusion into multinucleated osteoclasts, promoted the content of PDGF-BB, increased the quantity of type H vessels, and eventually takes precautions against OVX-induced bone loss in vivo.

Aucubin is derived from *Eucommia ulmoides*, a traditional Chinese medicine that has bone protection effects. As an iridoid glycoside compound, aucubin displays anti-inflammatory and antioxidative effects [26]. A recent study also demonstrated that aucubin regulates neovascularization in hindlimb ischaemia [19]. However, its effect on the bone is not as well understood. In the present experiments, we built an OVX model to detect the effect of aucubin on bone metabolism. Intraperitoneal injection of aucubin can significantly increase bone mass caused by OVX, which agreed with the outcomes reported by Li et al. [17, 18]. Meanwhile, the contents of PDGF-BB in bone marrow increased after aucubin intervention, suggesting that aucubin might increase bone mass through the enhancing angiogenesis induced by PDGF-BB. Further immunofluorescence results



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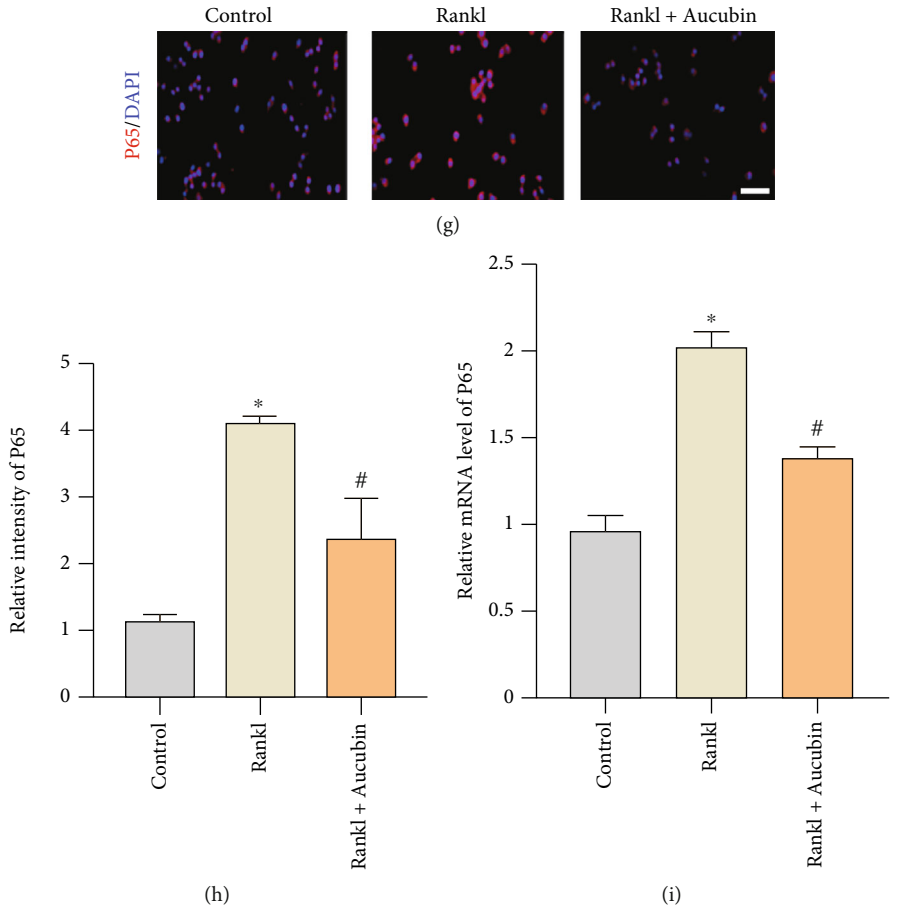

Figure 6: Aucubin inhibits the MAPK and NF-κB signaling pathways. (a) Protein levels of the MAPK family were analyzed by WBs. (b–d) Quantification of MAPK family protein expression. $^{*}P < 0.05$ compared to the Rankl group at 20 min; $^{\#}P < 0.05$ compared to the Rankl group at 30 min. (e) Protein levels of the NF-κB family were analyzed by WBs. (f) Quantification of NF-κB family protein level. $^{*}P < 0.05$ compared to the Rankl group at 20 min; $^{\#}P < 0.05$ compared to the Rankl group at 30 min. (g) Location of p65 fluorescence was observed via IF. Scale bar: 50 μm. (h) Relative p65 fluorescence intensity was analyzed. $^{*}P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the Rankl group. (i) Relative p65 mRNA was analyzed. $^{*}P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the Rankl group.

were consistent with these findings and showed that aucubin could upregulate the quantity of type H blood vessels characterized by CD31 and endomucin. Despite the low quantity, type H vessels undertake the process of bone formation by closely surrounded by osteoprogenitors. In addition to providing blood supply, type H vessels can secrete many factors that stimulate the proliferation and osteogenesis of osteoprogenitors. Bone loss related to ageing and bone diseases is at least partly resulting from the changes in quantity and function of type H vessels. Conform with previous researches, the present study also shows that the content of type H vessels is related to OVX-induced bone loss. Researches have shown that type H vessels play a vital role in the treatment of osteoporosis, fracture, and other bone-loss disease models [27, 28]. In the present study, aucubin attenuated the decreased OCN in serum and bone tissue caused by OVX and enlarged the number of type H vessels. This result shows that aucubin might be a candidate for osteoporotic treatment.

In the present study, aucubin alone did not increase angiogenesis in vitro indicating that the augmented angiogenesis caused by aucubin might due to an indirect effect. Furthermore, we found an upregulation of PDGF-BB in the CM after the administration of aucubin. And the remarkably enhanced angiogenic activities of MMECs were accompanied by the increase of PDGF-BB level in the cultured CM. Meanwhile, VEGF, the important marker of angiogenesis, was upregulated after the administration of aucubin+Rankl CM. Previous studies had shown that the levels of PDGF-BB and VEGF were strongly correlated [29]. PDGF is an important upstream mediator in hypoxia-induced VEGF up-regulation [30]. And PDGF-BB has been reported to induce the secretion of VEGF in a manner dependent on both Akt and MAPK activation in ovarian cancer [31]. The enhanced angiogenic activities induced by aucubin+Rankl CM were abolished by the intervention of PDGF-BB-neutralizing antibodies, confirming that the upregulation of angiogenic activities was induced

by PDGF-BB. Previous studies have confirmed that PDGF-BB could enhance the type H vessels angiogenesis and subsequent osteogenesis during bone remodeling [32]. PDGF-BB is an important member of the PDGF family which is important in the process of the proliferation, migration, and differentiation of endothelial progenitor cells. It exerts its function by binding to its specific receptor PDGF receptor β (PDGFRβ) mitogen-activated kinase and inducing a signaling cascade [33]. Recently, preosteoclasts have been confirmed as the most important source of PDGF-BB. In the present experiment, aucubin administration led to an accumulation of PDGF-BB in the CM as it increased the number of preosteoclasts. Further mechanism experiments showed aucubin increased the number of preosteoclasts by decreasing the expression of NFATc1, DC-STAMP, and ATPV0D2 mRNA induced by Rankl. NFATc1 is a vital regulator during the process of osteoclastogenesis. In addition to controlling the expression of the osteoclast differentiation related genes TRAP and cathepsin K, NFATc1 also participates in the multinucleation of osteoclasts through cell fusion molecules [34, 35]. DC-STAMP and ATPV0D2 participate in the process of cell-cell fusion [36, 37]. The fusion of macrophage cells into foreign body giant cells was completely abrogated in DC-STAMP-deficient mice [38]. In addition, knockdown of CTSK has been reported to inhibit the maturation of osteoclasts [10]. The decreased relative expression of osteoclast marker genes, including CTSK, DC-STAMP, ATP6V0D2, c-Fos, and NFATc1, demonstrated an inhibitory effect of aucubin on the differentiation of preosteoclasts into osteoclasts. The above results suggest that aucubin promotes type H vessel angiogenesis by inhibiting osteoclast fusion to produce more PDGF-BB.

After confirming that aucubin inhibited osteoclast fusion to promote type H vessel angiogenesis, we then explored the mechanism by which aucubin suppress preosteoclast fusion. In this research, we found that aucubin inhibited the activation of the MAPK signaling pathway. MAPK and NF-κB signaling pathways are vital during Rankl-induced osteoclast differentiation [39]. After Rankl administration, the activation of the MAPK family, namely, p38, JNK, and ERK, increased. The ERK signaling is involved in the survival, proliferation, and differentiation of osteoclasts [40]. Bone marrow-derived macrophages isolated which were lacking of JNK1 showed reduced osteoclast differentiation activity [41]. Activated p38 directly stimulates NFATc1 to enhance the differentiation of osteoclasts [42]. Meanwhile, aucubin was found to inhibit the phosphorylation and degradation of IKBα in the present study, which is an important part of NF-κB signaling pathway and suppresses p65 nuclear translocation by binding to p65. The IF staining results also indicated that aucubin inhibited the Rankl-induced activity of p65 signaling pathway. Recently, the PDGF-B promoter region was confirmed to contain an NF-κB binding domain [43]. Thus, the decrease in p65 activation into the nucleus directly inhibited the transcription of PDGF-BB. Taken together, we conclude that aucubin might enhance preosteoclast PDGF-BB-induced angiogenesis by inhibiting MAPK/NF-κB signaling and ultimately accelerate osteogenesis and prevent bone loss induced by OVX.

5. Conclusions

In conclusion, our research demonstrated that aucubin has the ability to inhibit multinucleated osteoclast maturation and promote the formation of type H vessels in OVX mice. The underlying mechanism may be that aucubin increases preosteoclast and subsequent PDGF-BB-induced angiogenesis by inhibiting MAPK/NF-κB signaling. All the findings indicate that aucubin might be an anti-bone-loss drug candidate which needs further research.

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 they have no conflicts of interest.

Authors' Contributions

Peng Xue and Sijing Liu designed the research; Ziyi Li, Chang Liu, and Xiaoli Liu carried out most of the experiments, generated data, and drafted the manuscript; Na Wang, Liu Gao, and Xiaoxue Bao helped to collect the samples; Peng Xue and Sijing Liu supervised the experiments and proofread the manuscript. Ziyi Li, Chang Liu, and Xiaoli Liu contributed equally to this work.

Acknowledgments

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Research Article

High-Fat Diet Increases Bone Loss by Inducing Ferroptosis in Osteoblasts

RunJiu Zhu,[1,2] ZhaoFu Wang,[3] Yuan Xu,[1,2] HaoYang Wan,[1,2] Xin Zhang,[1,4] MingRui Song,[1,2] Hong Yang,[1,5] Yu Chai,[1,2] and Bin Yu[1,2]

[1]*Division of Orthopaedic Surgery, Department of Orthopaedics, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China*
[2]*Guangdong Provincial Key Laboratory of Bone and Cartilage Regenerative Medicine, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China*
[3]*Department of Orthopaedics, People's Hospital of Ningxia Hui Autonomous Region, Yinchuan 750000, China*
[4]*Department of Orthopaedics & Traumatology, Wuyi Hospital of Traditional Chinese Medicine, Jiangmen 523000, China*
[5]*Department of Respiratory and Critical Care Medicine, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China*

Correspondence should be addressed to Yu Chai; ychaismu@163.com and Bin Yu; yubin@smu.edu.cn

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Current research suggests that chronic high-fat dietary intake can lead to bone loss in adults; however, the mechanism by which high-fat diets affect the development of osteoporosis in individuals is unclear. As high-fat diets are strongly associated with ferroptosis, whether ferroptosis mediates high-fat diet-induced bone loss was the focus of our current study. By dividing the mice into a high-fat diet group, a high-fat diet + ferroptosis inhibitor group and a normal chow group, mice in the high-fat group were given a high-fat diet for 12 weeks. The mice in the high-fat diet + ferroptosis inhibitor group were given 1 mg/kg Fer-1 per day intraperitoneally at the start of the high-fat diet. Microscopic CT scans, histological tests, and biochemical indicators of ferroptosis were performed on bone tissue from all three groups at the end of the modelling period. Mc3t3-E1 cells were also used in vitro and divided into three groups: high-fat medium group, high-fat medium+ferroptosis inhibitor group, and control group. After 24 hours of incubation in high-fat medium, Mc3t3-E1 cells were assayed for ferroptosis marker proteins and biochemical parameters, and osteogenesis induction was performed simultaneously. Cellular alkaline phosphatase content and expression of osteogenesis-related proteins were measured at day 7 of osteogenesis induction. The results showed that a high-fat diet led to the development of femoral bone loss in mice and that this process could be inhibited by ferroptosis inhibitors. The high-fat diet mainly affected the number of osteoblasts produced in the bone marrow cavity. The high-fat environment in vitro inhibited osteoblast proliferation and osteogenic differentiation, and significant changes in ferroptosis-related biochemical parameters were observed. These findings have implications for the future clinical treatment of bone loss caused by high-fat diets.

1. Introduction

Overweight and obesity are not only significant risk factors for cardiovascular disease, hypertension, and other cardiovascular diseases [1, 2] but also have adverse effects on bone through various mechanisms such as affecting bone formation and the bone marrow microenvironment [3]. The diseases arising from abnormal lipid metabolism and the development of osteoporosis are currently receiving increasing attention from researchers, who have found that patients with hyperlipidaemia can experience both bone loss and osteoporosis [4, 5]. At the same time, patients who develop osteoporosis and reduced bone mass also have abnormal lipid metabolism and vascular calcification [6], suggesting

that osteoporosis and lipid metabolism may be causally linked. Osteoporosis is a systemic skeletal disease characterized by a reduction in bone mineral density and bone strength, which increases the risk of fracture due to increased bone fragility [7]. The pathogenesis of osteoporosis is mainly due to a disruption of the balance between bone resorption and bone formation, resulting in a disorder of bone metabolism, which is associated with a decrease in the number and function of osteoblasts [8, 9]. The process of bone reconstruction is based on a dynamic balance between the two main mechanisms of osteoclast-associated bone resorption and osteoblast-associated bone formation [8]. And osteoblast cell death is an important pathogenic mechanism in osteoporosis [9]. Cell death is caused by a variety of mechanisms. The modes of cell death include autophagy, pyroptosis, senescence, and ferroptosis [10–12]. Osteoporosis is currently well studied in cellular senescence [13], cellular autophagy [14], and cellular pyroptosis [15], but less studied in cellular ferroptosis. Iron overload has been reported in the literature to cause osteoporosis [16, 17]. Therefore, controlling ferroptosis could be effective in preventing osteoporosis or promoting osteoblast osteogenic capacity. We therefore shifted our experimental focus to investigate the phenomenon of bone loss triggered by ferroptosis in osteoblasts. Ferroptosis was first proposed by Dixon et al. in 2012 [18] and is essentially the result of lipid peroxidation and excessive accumulation of intracellular reactive oxygen species due to damage to the cellular antioxidant system, which in turn leads to ferroptosis [19]. In ferroptosis, inhibition of glutathione peroxidase 4 and cystine/glutamate antiporter protein affects cysteine metabolism and promotes lipid peroxidation in cells [20]. Excess intracellular divalent iron causes ferroptosis in cells through the Fenton reaction [18]. In recent years, researchers have found that cells in high-fat environments are prone to ferroptosis [21, 22]. Ferroptosis plays an important role in cardiovascular diseases caused by high-fat diets [23, 24]. Therefore, it is reasonable to speculate that ferroptosis in osteoblasts in a high-fat environment is an important pathway for bone loss due to high-fat diets when mice are given a long-term high-fat diet. This holds great promise for the future clinical treatment of osteoporosis caused by high-fat diets.

2. Materials and Methods

2.1. Animals. The experimental protocol was approved by the Southern Hospital Animal Care and Use Committee and was conducted in accordance with the Southern Hospital Southern Medical University Guide for Laboratory Animals. The experimental mice were housed in a conventional experimental environment where the mice underwent a 12-hour light/12-hour dark cycle daily and were kept at a controlled temperature of around 25°C and 50% relative humidity with free access to food and drinking water. After one week of adaptive feeding, the mice were randomly assigned to three groups: normal control mice, high-fat diet mice, and high-fat diet + inhibitor mice. Mice in the high-fat diet group and mice in the high-fat diet + Fer-1 group were given HFD (D12492, Research Diet, USA) for a total of 12 weeks at the time of moulding. Mice in the high-fat diet + Fer-1 group were given a high-fat diet along with an intraperitoneal injection of 1 mg/kg Fer-1 (MCE, HY-100579, USA) starting daily. After the end of moulding, the mice were executed by the cervical dislocation method, and tissues were extracted for further experiments.

2.2. Micro-CT. After execution of the mice, the leg tissue was cut, and the femur was removed and washed in saline. This was followed by overnight fixation in 4% paraformaldehyde in a cold room at 4°C and studied using high-resolution CT scans (Skyscan 1172, Bruker MicroCT, Kontich, Belgium). The scans were performed using an X-ray energy of 55 kVp and a current of 145 mA, with a voxel size of 12 μm and an integration time of 400 ms. To get a clear picture of the changes in trabeculae in the region of the femoral epiphysis in mice, we used quantitative analysis which was performed using IPL software (Image Processing Language V5.15, Scanco Medical AG, Switzerland) for quantitative analysis. The region of the experiment (ROI) was selected starting 1 mm below the reference level of the distal epiphyseal plate and extending 2 mm in length in a distal direction for histomorphometric analysis of the trabeculae.

2.3. Histochemistry Staining. The femurs of the mice were separated from the soft tissue and fixed in 4% paraformaldehyde for 36 hours. This was followed by decalcification in 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0) for 10 days, followed by paraffin embedding. The resulting paraffin-embedded samples were cut longitudinally into 4 μm-thick sections. Alkaline phosphatase (ALP) staining and antitartrate acid phosphatase (TRAP) staining were performed, together with eosin (H&E) staining to visualize the histomorphology. To better assess the number of osteoblasts and osteoclast activity on the surface of bone trabeculae in experimental mice, the Wako TRAP/ALP stain kit (Cat. 294-67001) was used, and the manufacturer's instructions were followed: number of osteoblasts per millimeter of bone surface (N. of Ob/mm) and number of TRAP+ cells per millimeter of bone surface (N. of TRAP+). The number of osteoblasts per millimeter of bone surface (N. of Ob/mm) and the number of TRAP+ cells per millimeter of bone (N. of TRAP+/mm) were quantified.

2.4. Immunofluorescence Staining of Tissues and Cells

2.4.1. Immunofluorescence Staining of Tissues. The 4 μm paraffin wax was dewaxed and rehydrated into sodium citrate antigen repair solution and water bathed for 2 hours at 75°C, rinsed with PBS 3 times/min, and closed with BSA for one hour. This was followed by incubation with primary antibody GPX4 (Abcam, ab125066, 1 : 200) overnight at 4°C and then with fluorescently labelled secondary antibody for 1 hour at room temperature. Tissues were stained with anti-fluorescence-attenuated blocker containing DAPI as a counterstain (S2110, Solarbio, China). Sections were viewed with a Zeiss Axio Imager.D2 (Axio imager M2 Microscope, Germany). We randomly selected two nonoverlapping positions in three sections of each mouse specimen to quantify positive cells.

2.4.2. Immunofluorescence Staining of Cells. Cells were removed from the cell incubator after 24 hours of high-fat medium stimulation and Mc3t3-E1-E1 cells that had undergone seven days of osteogenic differentiation after high-fat medium stimulation, washed three times with PBS, and then, fixed with 4% paraformaldehyde for 15 minutes before washing the cells three times with PBS. The membrane was broken with 0.1% TritonX-100 for 15 minutes, followed by closure with 5% BSA for one hour. At the end of the closure high-fat medium was used to stimulate the cells for 24 hours after stimulation with primary antibodies Gpx4 (Abcam, ab125066, 1 : 50), Slc7a11 (Proteintech, 26864-1-AP, 1 : 50), and Ki67 (Abcam, ab15580, 1 : 50) to observe intracellular ferroptosis marker proteins and proliferation of changes. Cells undergoing seven-day osteogenic differentiation were treated with primary antibodies Osterix (Abcam, ab209484,1 : 50), Osteocalcin (Proteintech, 23418-1-AP, 1 : 50), and Rux2 (HUABIO, ET1612-47, 1 : 50) to observe changes in intracellular osteogenic-associated proteins. Cells were observed with a Zeiss Axio Imager.D2 (Axio imager M2 microscope, Germany).

2.5. Biochemical Analysis. The levels of reduced GSH/GSSG, total GSH (G263, Tong Ren, Japan), malondialdehyde (M496, Tong Ren, Japan), and divalent iron ions (l291, Tong Ren, Japan) in mouse bone tissue and Mc3t3-E1 cells were measured using commercial kits according to the manufacturer's instructions. Measurements were made using commercial kits, following the manufacturer's instructions.

2.6. Cell Culture. MC3T3-E1 (GNM15, Cell Bank of Typical Culture Preservation Committee of Chinese Academy of Sciences, China) cells were cultured in medium containing 10% FBS in α-MEM. To investigate whether osteoblasts under high-fat condition media (Pythonbio, AAPR156-D500, China) undergo ferroptosis and have reduced osteogenic capacity, we seeded MC3T3-E1 cells at a density of 1×10^5 cells/well on six-well plates. Cells were divided into control, high-fat, and high-fat plus Ferrostatin-1 groups at a concentration of 5 μmol/l. Cells were collected 24 h later for ferroptosis-related assays. For osteogenic differentiation, MC3T3-E1 cells were divided into control group, high-fat group, and high-fat plus Ferrostatin-1 group with Ferrostatin-1 (MCE, HY-100579, China) at a concentration of 5 μmol/l. After 24 hours of treatment in the high-fat and high-fat + Ferrostatin-1 groups, the medium was changed back to the conventional osteogenic differentiation induction medium. On the seventh day, ALP staining and immunofluorescence staining for bone-formation-associated proteins were performed, and the results of ALP staining were quantified.

2.7. Osteogenic Differentiation of Cells and ALP Staining. Alkaline phosphatase (ALP) staining assays were used to assess the effect of inhibition of cellular ferroptosis under high-lipid conditions on osteogenic differentiation of Mc3t3-E1cells. Mc3t3-E1 cell suspensions were added to 24-well plates (2×10^4 cells per well) and incubated in a conventional incubator for 12 hours. The medium was then changed to regular medium, high-fat medium, and high-fat plus Ferrostatin-1 medium. After 24 hours of incubation, all media were changed to osteogenic differentiation induction medium, which was prepared with 50 μg/ml ascorbic acid (Sigma, A4544-25G), 10 nmol/l dexamethasone (MCE, HY-14648), and 10 mmol/l β-glycerophosphate (Sigma, G9422-10G). Seven days after osteogenic induction, half of the cells were stained for ALP using the ALP staining kit for the plates (Beyotime, C3206), and staining was performed according to the kit guidelines. Observation was made with a bright-field microscope. Photographs were then taken with a bright-field microscope ((OLYMPUS, BX63). The other half was assayed for ALP activity using the ALP quantification kit. The supernatant was collected, and the absorbance was measured at 405 nm (Beyotime, P0321S), and the cellular ALP activity was counted quantitatively.

2.8. Statistics. All quantitative data were expressed as mean ± S.E.M. For cell culture experiments, all results were obtained from independent replicates of the experiments, which were repeated independently at least three times. For comparisons between two groups, independent Student's t-tests were used. One-way analysis of variance (ANOVA) and Bonferroni post hoc tests were used for multiple comparisons. Statistical analysis software was used for the data using GraphPad, version 7.0 software (GraphPad Software, USA). P values < 0.05 considered to be a statistically significant difference.

3. Results

3.1. Ferroptosis Inhibitor Ferrostatin-1 Can Prevent HFD-Induced Bone Loss. To research the efficacy of Ferrostatin-1 on bone formation and bone resorption in high-fat diet mice, we used a model of bone loss induced by a long-term high-fat diet. Mice fed a high-fat diet were given intraperitoneal injections of Ferrostatin-1, and the results showed that the administration of a high-fat diet to mice resulted in a reduction in femoral trabecular bone mass. This high-fat diet-mediated bone loss was inhibited by Ferrostatin-1. Morphometric analysis of the distal femur showed that BV/TV and number (Tb.N) were reduced and that this change was inhibited by Ferrostatin-1. However, the effect of Ferrostatin-1 on trabecular bone thickness (Tb.Th) but trabecular bone separation (Tb.Sp) in mice on a high-fat diet was not statistically significant (Figure 1(d)).

3.2. High-Fat Diet Induces Loss of Osteoblasts. As a result of the analysis of micro-CT data, a chronic high-fat diet alters the bone structure of long bones in mice. We investigated in our experiments whether the bone marrow microenvironment of HFD-fed mice leads to a decrease in the number of osteoblasts. It was again verified by histological examination of HE staining that bone loss in high-fat diet mice could be mitigated by Ferrostatin-1, as shown by H&E staining (Figure 2(a)). During the course of our study, we identified an important role for the precursors of osteoblast formation. Proosteoblasts are an important population for bone formation in the bone marrow cavity. In order to detect changes in osteoblasts on the surface of bone trabeculae. ALP staining

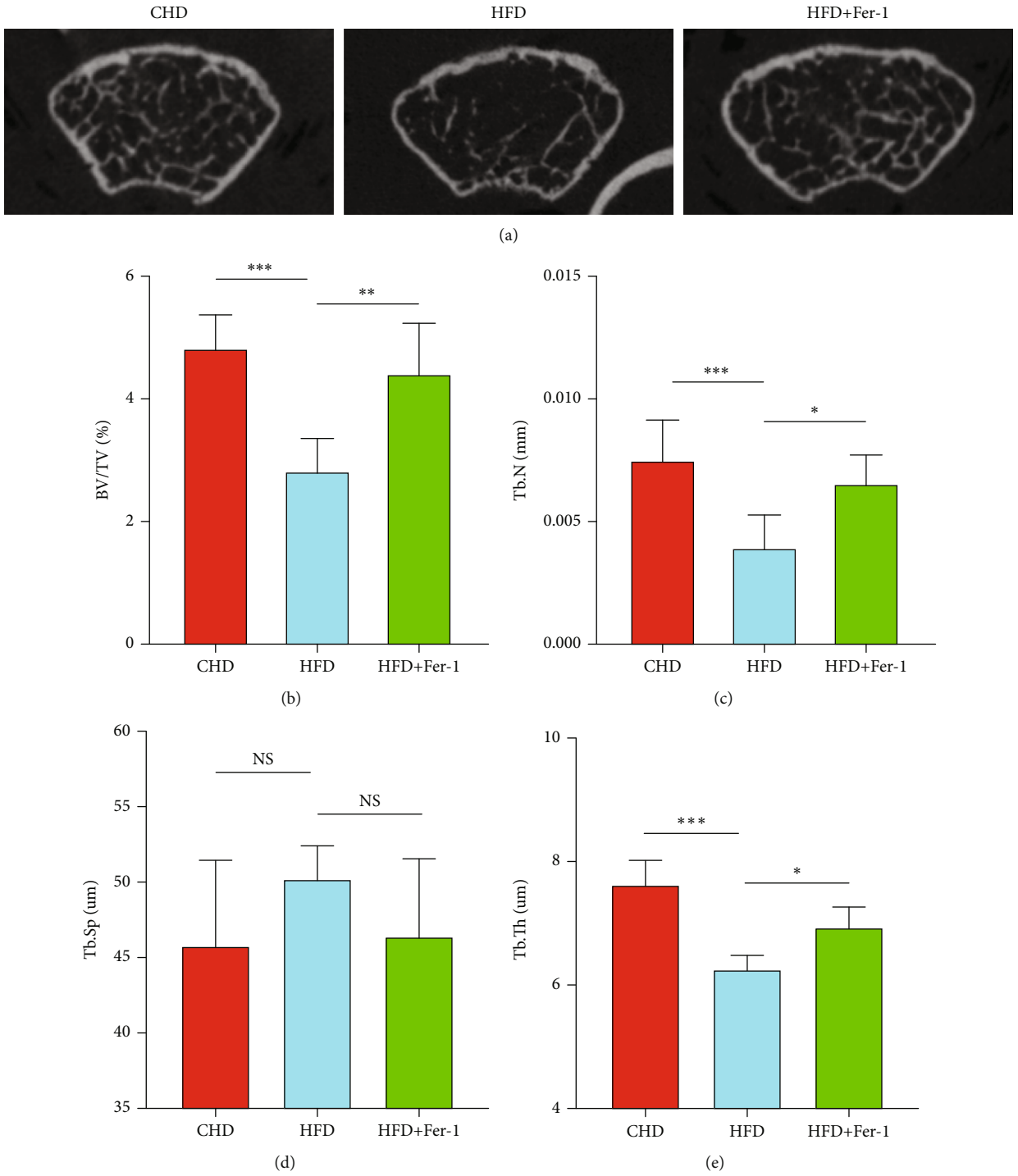


Figure 1: In this experiment, it was found that feeding mice a high-fat diet reduced bone mass production and that intraperitoneal administration of the ferroptosis inhibitor Ferrostatin-1 to mice fed a high-fat diet significantly reduced the bone mass loss in mice fed a high-fat diet. Representative micro-CT images of the distal femur of mice fed a normal diet, a high-fat diet, and a high-fat diet plus a ferroptosis inhibitor for 12 weeks (a). The femoral microstructure of mice was then quantified in terms of trabecular volume fraction (BV/TV) (b), trabecular number (Tb.N) (c), trabecular thickness (Tb.Sp) (d), and trabecular separation (Tb.Th) (e). $n = 5$/group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

was used to quantify the number of osteoblasts in the experiment (Figures 2(a) and 2(b)). The results showed that the number of osteoblasts on the surface of bone trabeculae was reduced in high-fat diet mice and that Ferrostatin-1 inhibited the reduction in their number, since the metabolic balance of bone is maintained by a balance between osteoclast formation and bone resorption. For this reason, bone tissue was experimentally stained with TRAP (Figures 2(a)

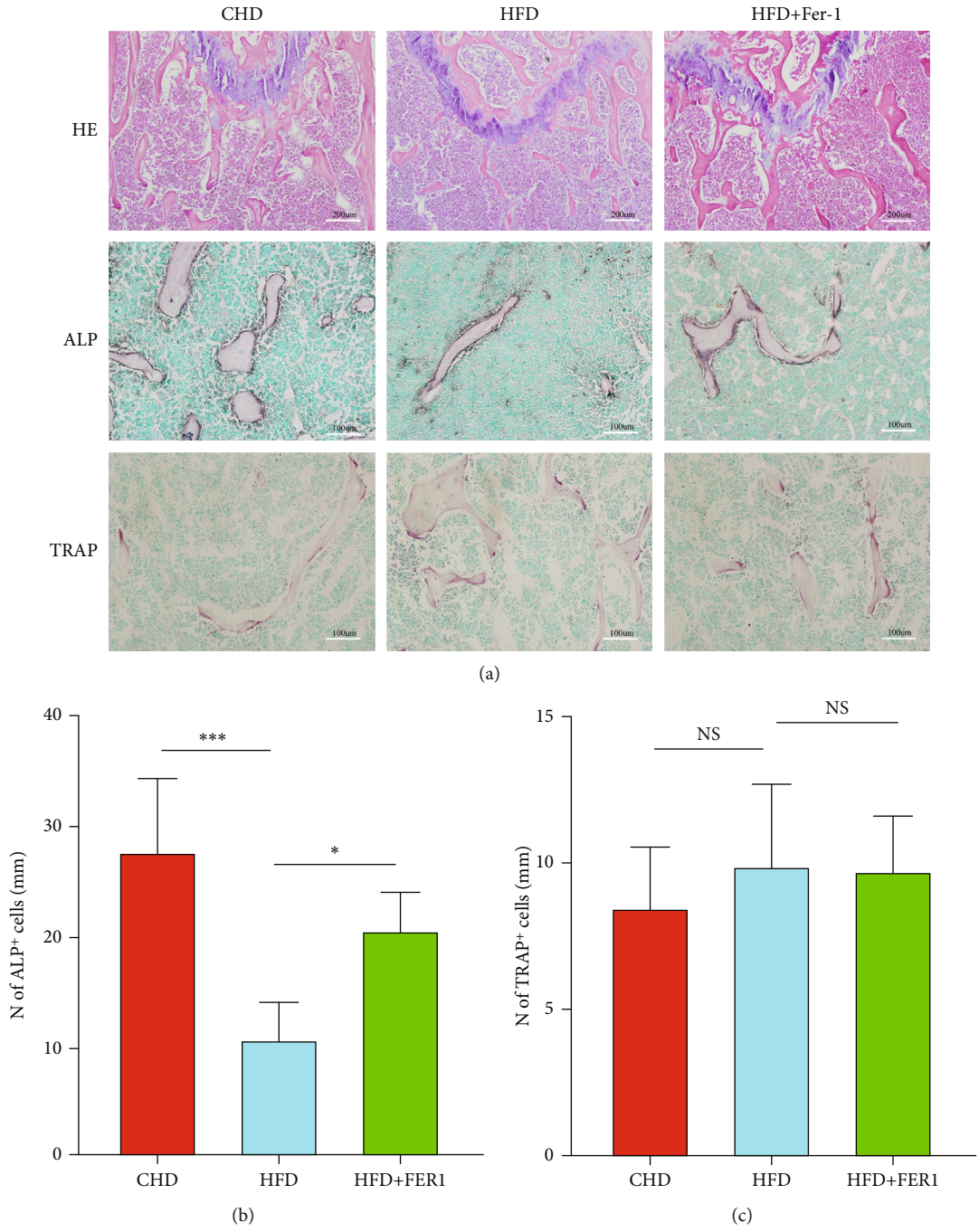
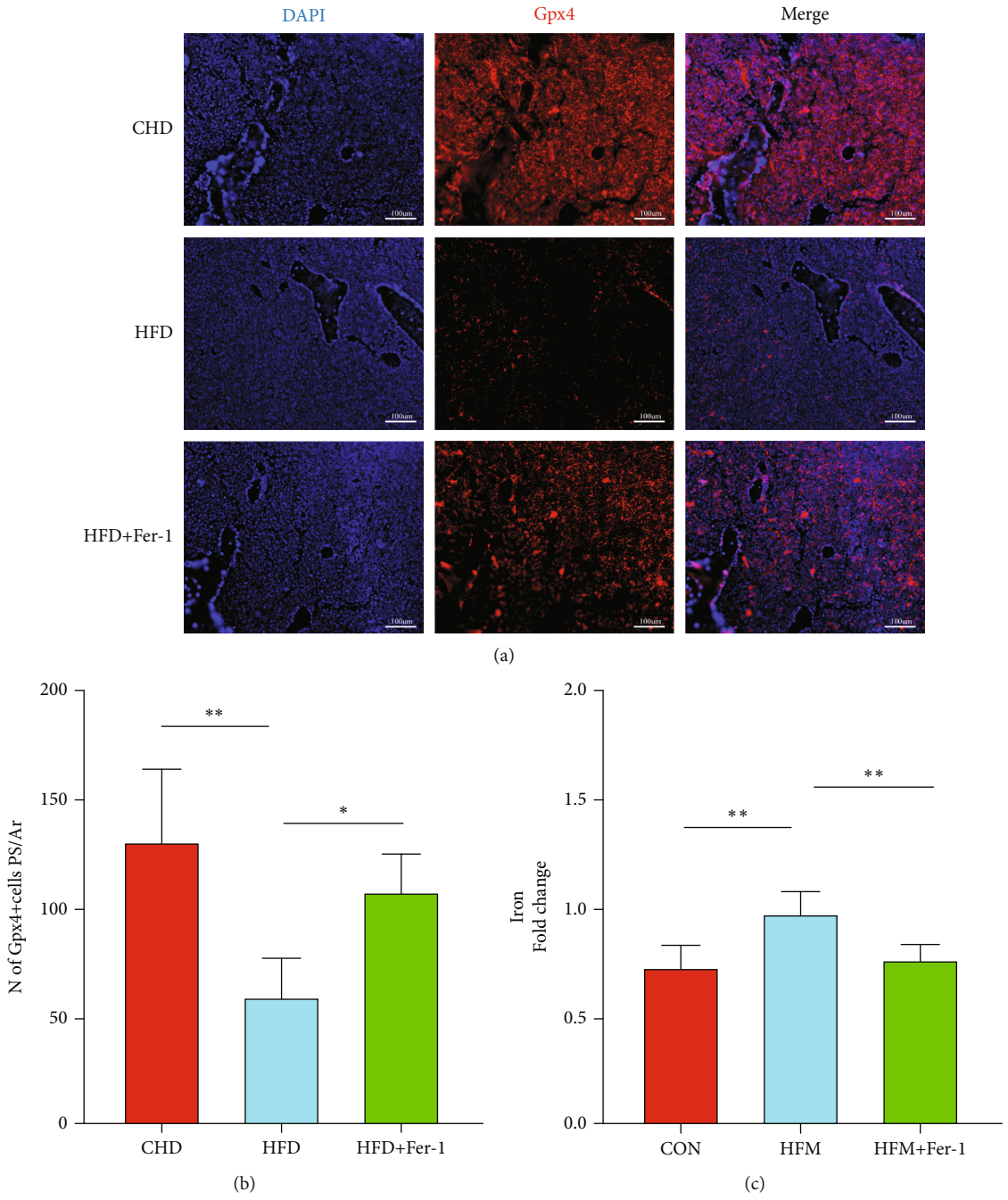


Figure 2: Chemical staining of mouse femurs with hematoxylin and eosin (H&E) after 12 weeks of feeding mice on normal and high-fat diets revealed a significant reduction in the number of metaphyseal trabeculae in the femur, which was then restored to some extent after administration of ferroptosis inhibitors (a); scale bar represents 200 μm. Experiments revealed that after mice consumed a diet high in fat content, the number of alkaline phosphatase- (ALP-) positive osteoblasts on the surface of the trabeculae in the femur was significantly reduced (b), and the reduction in the number of osteoblasts was somewhat mitigated by a high-fat diet accompanied by treatment with ferroptosis inhibitors in mice. Osteoblasts are shown in brown and nuclei in green. Quantification of the number of osteoblasts per bone surface (b). Scale bars represent 100 μm, $n = 5$/group, $^{*}P < 0.05$, $^{***}P < 0.001$. Quantification of staining for TRAP+ cells in the femoral bone marrow cavity (a, c), with osteoclasts in red and nuclei in green (a) and NS indicating no statistical difference in component; scale bars represent 100 μm, $n = 5$/groups.

and 2(c)) to assess changes in osteoclast activity in the bone marrow cavity of mice on a high-fat diet. However, we observed no significant difference in the number of TRAP^{+} cells in the bone of mice given a high-fat diet. Therefore, these results suggest that a high-fat diet may primarily induce the loss of osteoblasts in mice.



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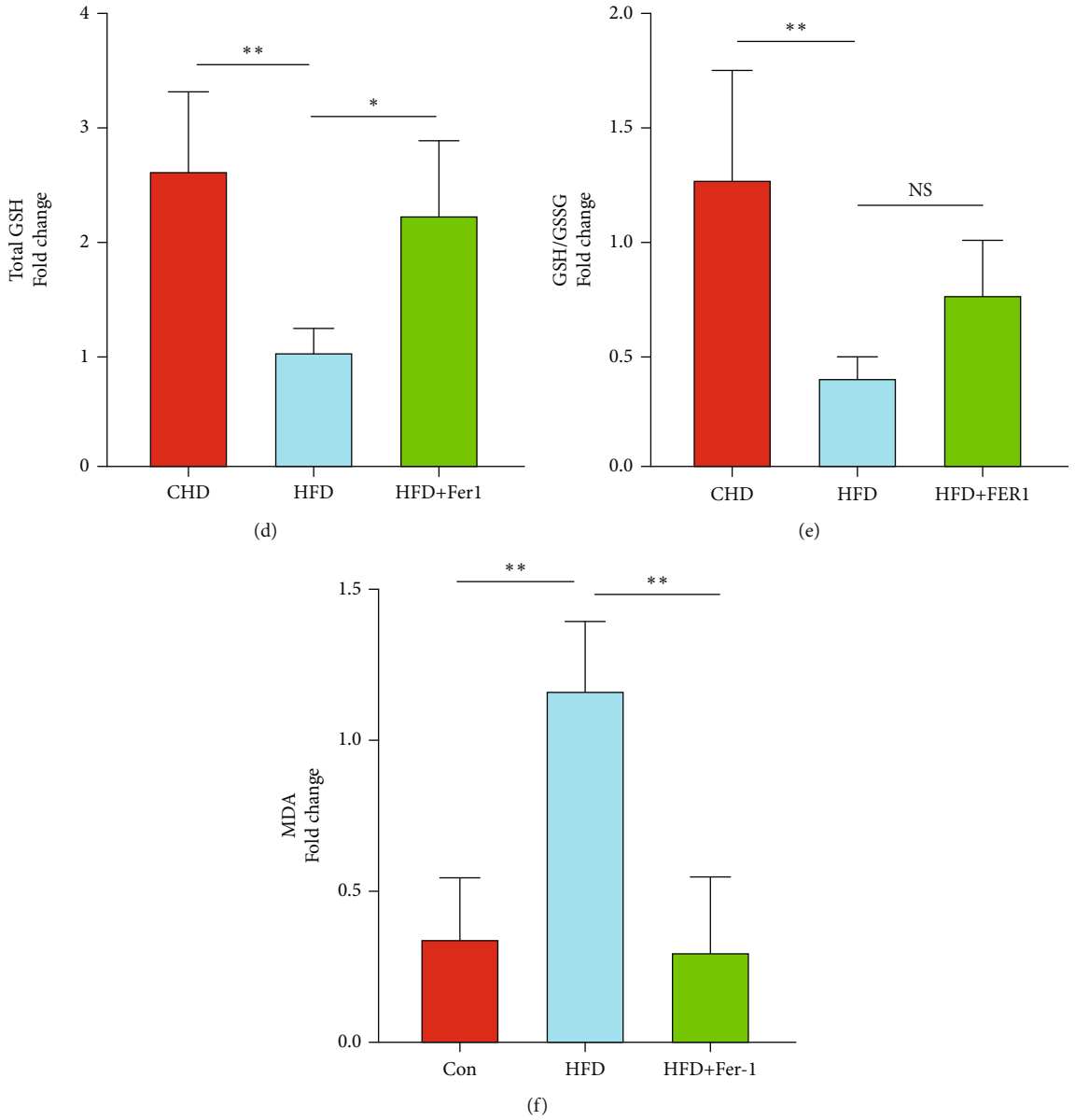
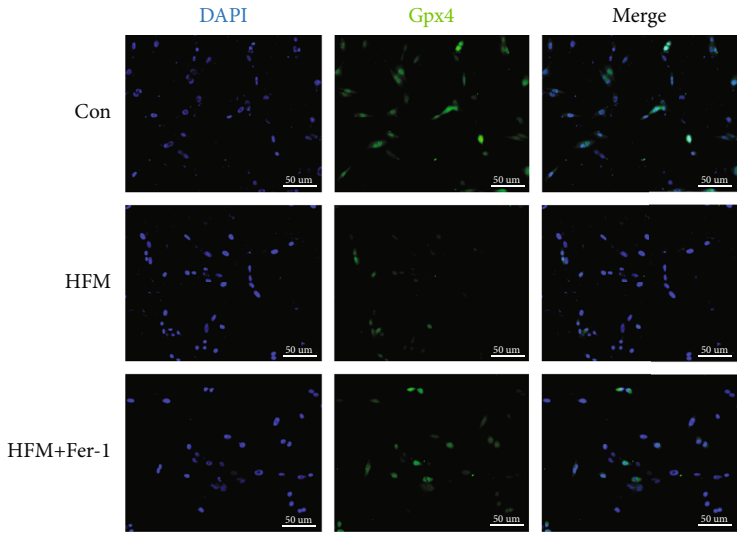


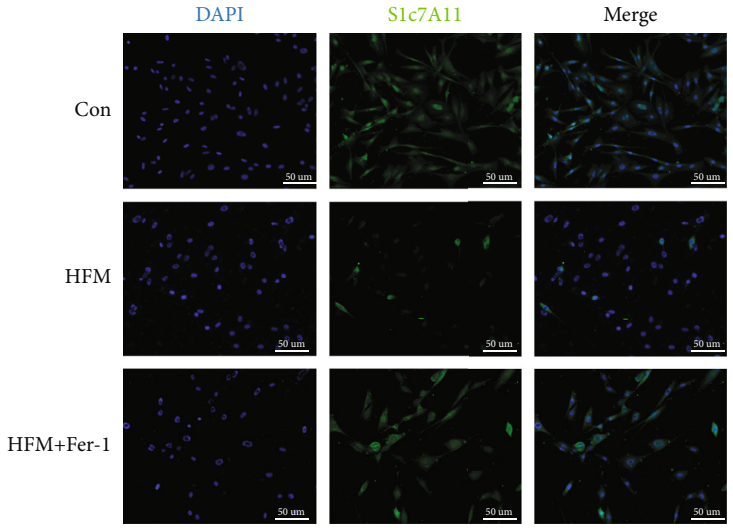
Figure 3: To demonstrate whether ferroptosis occurred in bone tissue of mice fed a high-fat diet, the expression of Gpx4 protein, a key protein for ferroptosis, in the femur of mice was examined using immunofluorescence staining. A significant decrease in the number of Gpx4 positive cells in the bone marrow of mice fed a high-fat diet was found (a), and after administration of a ferroptosis inhibitor, the number of Gpx4-positive cells increased significantly compared to the group fed a high-fat diet alone (a, b). Gpx4-positive cells in red and DAPI-stained nuclei in blue. $n = 5$/group, $^{*}P < 0.05$, $^{**}P < 0.01$. By measuring biochemical indicators of ferroptosis in the normal diet group, high-fat diet group, and high-fat diet plus ferroptosis inhibitor group, iron content (c), the total GSH content (d), GSH/GSSG ratio (e), and MDA content (f) in the femurs of mice were found to be $n = 5$/group, $^{*}P < 0.05$, $^{**}P < 0.01$; NS indicates no statistical difference.

3.3. Ferroptosis Plays an Important Role in High-Fat Diet-Induced Bone Loss. In previous studies, researchers found that Gpx4, a key protein for ferroptosis, is significantly altered in a high-fat environment. Immunofluorescent staining of bone tissue from mice on a high-fat diet suggested that Gpx4 protein expression was decreased in the bone marrow lumen and that this process could be reversed by Ferrostatin-1 (Figure 3(a)). The tibial bone tissues of mice were also subjected to the determination of biochemical indicators of ferroptosis, namely, total GSH, GSH/GSSG ratio, MDA, and Fe2+ content. The results showed that these MDA and Fe2+ were significantly elevated in the bone tissues of mice on a high-fat diet. However, total GSH and GSH/GSSG ratios were significantly lower. These data suggest that ferroptosis plays an important role in the high-fat diet-induced bone loss.

3.4. Ferroptosis Is an Important Way in Which Mc3t3-E1 Cells Undergo Death. Years of research have shown that osteogenic differentiation of Mc3t3-E1 cells is important



(a)



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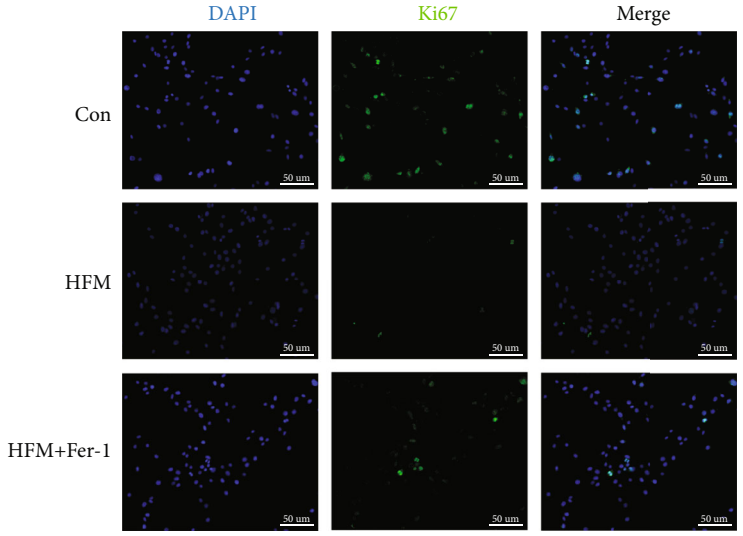

(c)

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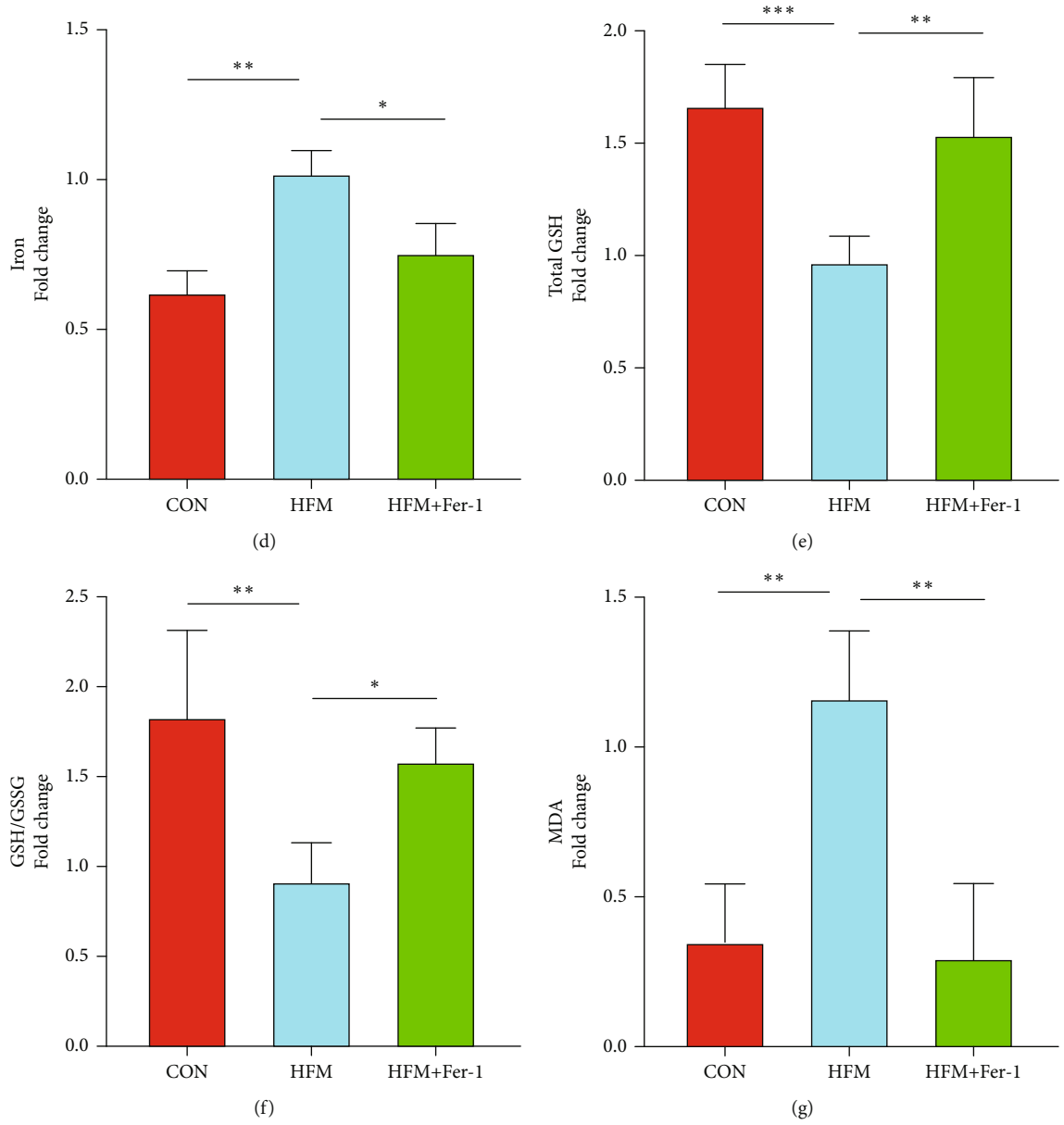
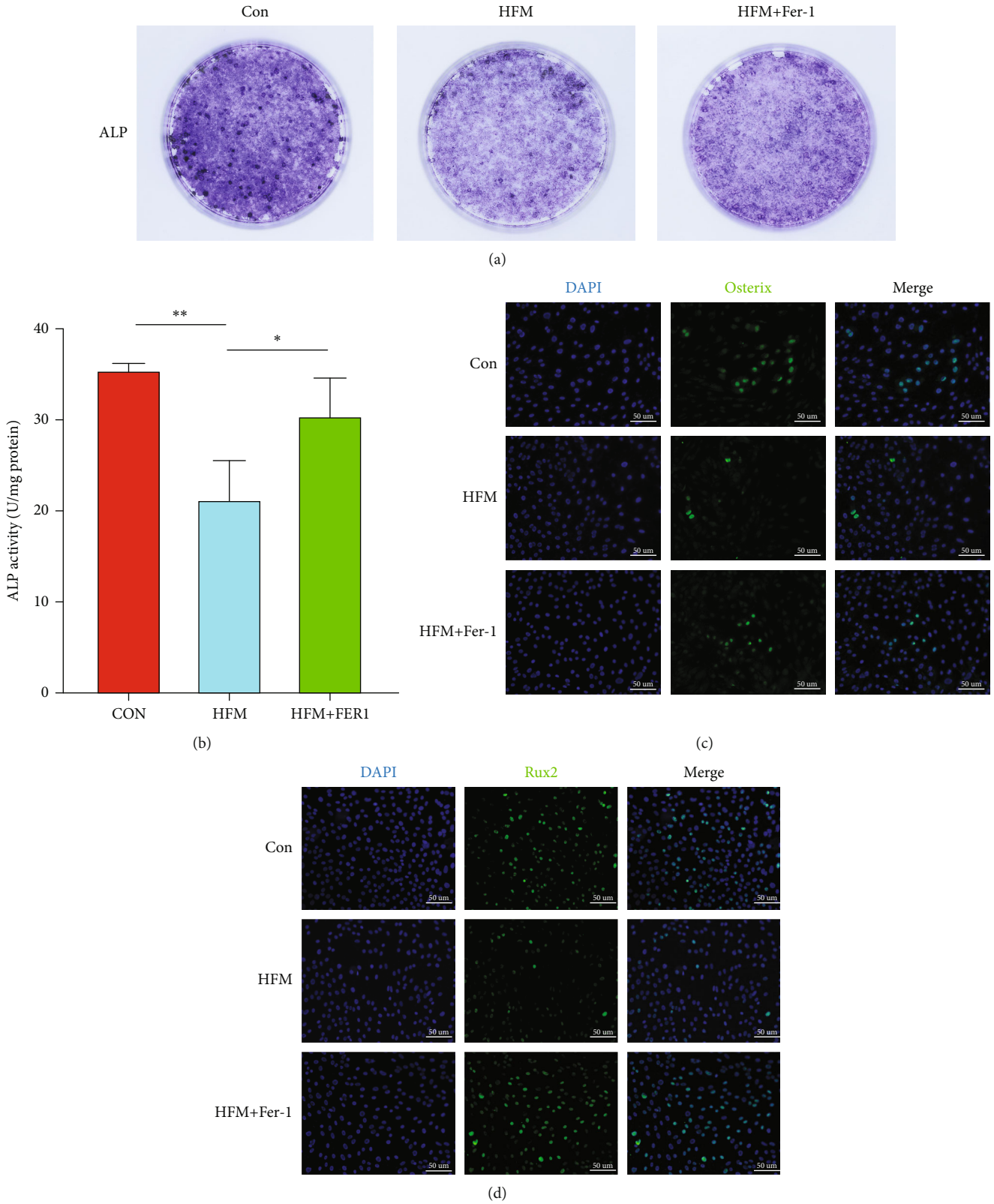


Figure 4: Mc3t3-E1 cells were used as cells for the in vitro study. Mc3t3-E1 cells were treated with high-fat medium for 24 hours, and Fer-1 at a concentration of 5 μmol/l was used to slow down the process of ferroptosis. 24 hours later, changes in the key ferroptosis proteins Gpx4 and Slc7a11 were measured (a, b), while there was also a significant decrease in cell proliferation capacity (c), a process that could be rescued by ferroptosis inhibitors. Some of the treated cells were also used to assay ferroptosis biochemical parameters, and intracellular iron (d), total GSH (e), GSH/GSSG (f), and MDA (g) were found to vary in the high-fat medium. $n = 3$/group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

for bone formation. If ferroptosis of Mc3t3-E1 cells is increased in a high-fat environment, its function and proliferative capacity will be greatly affected. To determine whether Mc3t3-E1 undergoes ferroptosis under high-fat conditions, immunofluorescence staining for Gpx4 and Slc7a11, the key proteins of ferroptosis, and biochemical indicators of ferroptosis, was performed. The results revealed that total GSH and total GSH/GSSG ratio were lower in Mc3t3-E1 cells treated with high-fat medium compared to the control (Figures 4(e) and 4(f)), while Fe2+ and MDA were higher in Mc3t3-E1 treated with high-fat medium (Figures 4(d) and 4(g)) and increased, and Gpx4 and Slc7a11 proteins were significantly reduced compared to the control. Moreover, the proliferation function of Mc3t3-E1 cells was somewhat restricted compared to the control group. This process could be inhibited by Ferrostatin-1. These results suggest that ferroptosis is present in Mc3t3-E1 cells cultured in a high-fat environment.

3.5. High-Fat Environment Inhibits Osteogenic Differentiation of Mc3t3-E1 Cells. Although cell death is an important factor affecting changes in bone mass in mice, impaired osteogenic differentiation of Mc3t3-E1 cells has a major impact on reduced bone mass in mice. Therefore,



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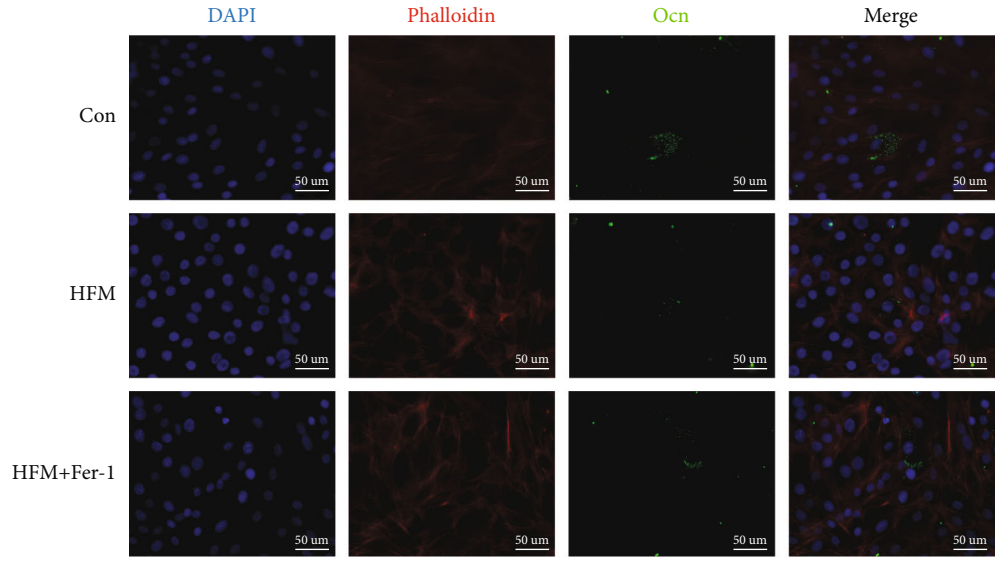

(e)

Figure 5: To verify that osteogenic formation is affected to some extent in a high-fat environment. We treated Mc3t3-E1 cells in high-fat medium and then replaced the osteogenic induction medium for seven days of osteogenic differentiation culture. We found that the level of alkaline phosphatase ALP was significantly reduced in high-fat conditioned medium-treated cells at day seven of osteogenic induction culture (a, b) and that this process could be alleviated by ferroptosis inhibitors. $n = 3$/group, $^{*}P < 0.05$, $^{**}P < 0.01$. Early intracellular osteogenic differentiation was measured by immunofluorescence at day seven of osteogenic differentiation in high-fat-treated cells Osterix (c), Rux2 (d), and Ocn (e) found to be reduced to varying degrees. The addition of Fer-1, a ferroptosis inhibitor, to the high-fat medium treatment significantly reduced the expression of osteogenic marker proteins (c–e).

the osteogenic differentiation capacity of Mc3t3-E1 cells was examined in this experiment. During osteogenic differentiation of the cells, we found that the early marker of osteogenic differentiation, ALP, in Mc3t3-E1 cells treated with high-fat medium was significantly decreased at the seventh day of osteogenic differentiation induction (Figures 5(a) and 5(b)). The osteogenic-related proteins Osterix, Osteocalcin, and Rux2 were also reduced to varying degrees (Figures 5(c)–5(e)). And this process could be inhibited by Ferrostatin-1.

4. Discussion

Chronic obesity caused by a high-calorie diet has a significant negative impact on human bone mineral density (BMD) [25]. The long-term reduction in BMD and consequently osteoporosis increases the economic burden on society [26]. However, the exact mechanism of bone loss caused by a high-fat diet is not known [25]. Therefore, the search for the exact mechanism of bone loss caused by a high-calorie diet is of great importance for the prevention and treatment of bone loss. The essence of cellular ferroptosis is that lipid peroxidation and reduced expression of the core proteins Gpx4 and Slc7a11 occur in response to external stimuli that impair the cellular antioxidant system, which also affects the metabolism of cystine transport [27, 28]. In previous studies, the link between the occurrence of cellular ferroptosis and abnormal cellular function in a high-fat environment has been inextricably linked. The association between high-fat environments and cellular ferroptosis is mainly related to cardiovascular aspects [29], such as arterial lipid deposition leading to plaque formation [30], cardiac ejection capacity [31], and remodelling [32], but the mechanisms are underlying alcoholic liver disease [33]. From these studies, we can suggest that there is a link between a high-fat diet and the development of cellular ferroptosis. Therefore, it is reasonable to assume that the phenomenon of cellular ferroptosis plays an important role in the loss of bone mass in mice on a high-fat diet. In a high-fat environment, most cells undergo accumulation of lipids and excessive accumulation of reactive oxygen species [34, 35]. These are necessary conditions for ferroptosis to occur. Therefore, inhibition of ferroptosis is important in the process of impaired cellular function caused by high-fat environments. In the present experiment, mice on a high-fat diet injected with Ferrostatin-1, a ferroptosis inhibitor, showed a significant reduction in bone loss (Figure 1). In vivo experiments have confirmed the important role of ferroptosis in bone loss due to a high-fat diet. In the present study, the high-fat diet led to bone loss in mice mainly due to a disruption of the balance between osteogenesis and osteolysis, with a low number of osteoblasts, resulting in impaired osteogenic mineralization and reduced bone formation (Figure 5). In the present study, we first demonstrated in vivo experiments in animals that ferroptosis is an important influence on bone loss due to a high-fat diet. Following in vivo experiments, we found that impaired bone anabolism was an important factor in the high-fat diet-induced bone loss. The biochemical parameters of ferroptosis in mouse bone tissues also confirmed that ferroptosis occurs in the process of bone loss due to high-fat diet (Figure 3). Since osteogenic differentiation of osteoblasts is the cell that most directly affects bone synthesis, in the

next study, we used in vitro culture of osteoblasts (Mc3t3-E1 cells) to verify whether bone formation of osteoblasts is affected to some extent in a high-fat environment and that cellular ferroptosis is an important factor affecting osteogenic differentiation of osteoblasts in a high-fat environment. The results of the cellular assays suggest that the high-fat environment does indeed diminish osteoblast proliferation (Figure 4) and osteogenic differentiation (Figure 5) and that this process can be mitigated by ferroptosis inhibitors. The results of this in vitro experiment are consistent with those of the in vivo experiments. It confirms that ferroptosis does occur in the high-fat environment and attenuates the normal physiological functions of the cells. The results of this study may provide a possible treatment for the reduction in bone mineral density caused by a high-fat diet. However, there are several shortcomings in this study. Firstly, a high-fat diet does not only affect the growth and development of bones but also other important organs to varying degrees. Whether damage to other important organs may indirectly lead to bone loss was also not investigated in this study. For example, it has been established that lipid deposition due to a high-fat diet has an adverse effect on blood vessels throughout the body and that the H-vessel, which represents bone anabolism, may also be significantly reduced, thereby reducing the supply of nutrients to the femoral epiphysis and indirectly leading to bone loss. Secondly, other cells that represent bone anabolism were not targeted and analyzed in this experiment, such as whether the number and function of bone marrow mesenchymal stem cells, which have the potential for multidirectional differentiation, were affected in some way and thus were a factor in bone loss in mice on a high-fat diet. In future studies, we will explore the clinical translation of cellular ferroptosis in bone loss due to high-fat diet in an effort to investigate the underlying mechanisms.

5. Conclusions

In the present experiment, we found that long-term high-fat diet intake led to bone loss in mice by feeding them a high-fat diet (a). And cellular ferroptosis was involved in the whole process. The results suggest that impaired bone anabolism is an important factor in bone loss in mice fed a high-fat diet (b). The reduced osteoblast differentiation function under high-fat conditions is a major factor affecting bone formation. Previous studies have found that high body weight due to high calories is an important factor contributing to bone loss in people. As people's standard of living has now improved and their diet has changed, long-term high calorie intake can lead to damage to bone microstructure. Therefore, the high-fat diet found in this experiment interferes with normal bone anabolic metabolism through the ferroptosis pathway has some clinical significance. The possible pathways by which a high-fat diet leads to bone loss are described from a mechanistic perspective. The antagonism of ferroptosis through the administration of antioxidant drugs to slow down bone loss due to high-fat diet may also be a potential clinical treatment for bone loss due to high-fat diet.

Data Availability

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

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

RunJiu Zhu, ZhaoFu Wang, and Yuan Xu contributed equally to this work. RunJiu Zhu drafted the manuscript; RunJiu Zhu, ZhaoFu Wang, Yuan Xu, and Hong Yang drew the picture. HaoYang Wan, MingRui Song, and Xin Zhang drew figure notes; Bin Yu and Yu Chai supervised the research and revised the manuscript.

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Research Article

Mesenchymal Stem Cells May Alleviate the Intervertebral Disc Degeneration by Reducing the Oxidative Stress in Nucleus Pulposus Cells

Yongzhao Zhao,[1,2,3] **Qian Xiang**,[1,2,3] **Yunzhong Cheng**,[4] **Jialiang Lin**,[1,2,3] **Shuai Jiang**,[1,2,3] **and Weishi Li**[1,2,3]

[1]*Department of Orthopaedics, Peking University Third Hospital, Beijing, China*
[2]*Beijing Key Laboratory of Spinal Disease Research, Beijing, China*
[3]*Engineering Research Center of Bone and Joint Precision Medicine, Ministry of Education, Beijing, China*
[4]*Department of Orthopedic Surgery, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China*

Correspondence should be addressed to Weishi Li; puh3liweishi@163.com

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Background. Stem cell therapy is a promising therapeutic modality for intervertebral disc degeneration (IDD). Oxidative stress is a vital contributor to the IDD; however, the definite role of oxidative stress in stem cell therapy for IDD remains obscure. The aim of this study was to determine the vital role of oxidative stress-related differentially expressed genes (OSRDEGs) in degenerative NPCs cocultured with mesenchymal stem cells (MSCs). *Methods*. A series of bioinformatic methods were used to calculate the oxidative stress score and autophagy score, identify the OSRDEGs, conduct the function enrichment analysis and protein-protein interaction (PPI) analysis, build the relevant competing endogenous RNA (ceRNA) regulatory networks, and explore the potential association between oxidative stress and autophagy in degenerative NPCs cocultured with MSCs. *Results*. There was a significantly different oxidative stress score between NPC/MSC samples and NPC samples ($p < 0.05$). Forty-one OSRDEGs were selected for the function enrichment and PPI analyses. Ten hub OSRDEGs were obtained according to the PPI score, including JUN, CAT, PTGS2, TLR4, FOS, APOE, EDN1, TXNRD1, LRRK2, and KLF2. The ceRNA regulatory network, which contained 17 DElncRNAs, 240 miRNAs, and 10 hub OSRDEGs, was constructed. Moreover, a significant relationship between the oxidative stress score and autophagy score was observed ($p < 0.05$), and 125 significantly related gene pairs were obtained ($|r| > 0.90$, $p < 0.05$). *Conclusion*. Stem cell therapy might repair the degenerative IVD via reducing the oxidative stress through the ceRNA regulatory work and restoration of autophagy in degenerative NPCs. This research could provide new insights into the mechanism research of stem cell therapy for IDD and potential therapeutic targets in the IDD treatment.

1. Background

Low back pain (LBP) has become a very common health concern in the modern society, which generates a social and economic burden to human beings [1–3]. It is estimated that approximately 80% of population experience the LBP at least once in their lifetimes [4]. Intervertebral disc degeneration (IDD) is the principal contributor to the LBP [2, 5]. IDD is an inflammatory-catabolic process triggered by a series of pathogenic factors, including gene susceptibility, increased mechanical stress, abnormal immunity, metabolic disorders, and oxidative stress [3, 6, 7]. The standard treatments for LBP caused by IDD include the bed rest, administration of nonsteroid anti-inflammatory drugs and analgesics, discectomy, and lumbar interbody fusion [2, 5]. However, the existing treatments can only relieve the clinical symptoms instead of reversing the degeneration process. Therefore, novel therapies targeting the degeneration process are urgently needed.

In recent years, stem cell therapy has shown a promising effect and potential clinical applicability in the management of IDD [8–12]. Accumulating evidence has indicated that mesenchymal stem cells (MSCs) might exert therapeutic functions mostly through the paracrine process, such as the release of growth factors, cytokines, extracellular vesicles, and noncoding RNAs [8]. However, the definite underlying mechanisms remain unclear. Oxidative stress has been demonstrated to play important roles in the development of IDD [13, 14]. Under normal circumstances, the microenvironment of intervertebral disc (IVD) tissue is hypoxic, and there is a dynamic balance between the generation and scavenging of intracellular reactive oxide species [15]. However, the oxidative stress occurs when this balance is disrupted, which can lead to senescence and apoptosis of nucleus pulposus cells (NPCs), and degradation of extracellular matrix [16].

Long noncoding RNAs (lncRNAs) refer to a type of noncoding RNA longer than 200 nucleotides [17, 18]. Although lncRNA lacks the ability to encode proteins, lncRNAs can act as the competing endogenous RNAs (ceRNAs) by sponging the microRNAs (miRNAs) to repress the translation of genes [19, 20]. Autophagy is a well-known conserved cellular process through which cells can realize the self-protection by scavenging the unwanted senescent organelles and misfolded proteins [21, 22]. The dysregulation of autophagy has been proved to associate with the development of several human diseases, including the IDD [21, 23]. Previous studies have shown that the oxidative stress could be relieved by activating the autophagy in degenerative NPCs, thereby reducing the apoptosis and degradation of extracellular matrix [22, 24, 25]. Nevertheless, to our knowledge, few articles focus on the effects of MSCs on the alleviation of oxidative stress via regulating the autophagy in IDD.

With the huge improvement of sequencing techniques, many key genes and noncoding RNAs associated with the IDD have been determined using the bioinformatic approaches [13, 26, 27]. We previously reported that oxidative stress is an important pathogenic factor for IDD [13]. Wang et al. found that infiltrating macrophages play important roles in the pathogenesis of IDD [26]. In Li et al. study, 305 genes closely related to IDD were obtained, and the authors also reported that DNA repair, oxidative phosphorylation, peroxisome, IL-6-JAK-STAT3 signaling, and apoptosis contributed to the development of IDD [27]. However, few bioinformatic analysis focusing on the role of stem cell therapy in the management of IDD are published to date. Hence, this study was conducted to explore the underlying mechanisms of stem cell therapy in the management of IDD using the strict and mature bioinformatic algorithms based on the relevant sequencing data.

2. Materials and Methods

This study has been approved by the Ethics Committee of Peking University Third Hospital, and the informed consent was not necessary because all data was obtained from public databases. The flow chart of this study has been shown in Figure 1.

2.1. Data Collection and Processing. Gene expression data of mRNAs and lncRNAs in GSE112216 was downloaded from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). This dataset contained the gene chip sequencing data of 3 NPC/MSC samples and 3 NPC samples, and compare the mRNA and lncRNA expression of degenerative NPCs cocultured with adipose-derived MSCs with degenerative NPCs solely. The oxidative stress-related gene (OSRG) list was extracted from the Gene Set: GOBP_RESPONSE_TO_OXIDATIVE_STRESS in Molecular Signatures Database (http://www.gsea-msigdb.org/gsea/msigdb/index.jsp) [26] (Supplementary Table 1). Besides, the autophagy-related gene list was obtained from the Human Autophagy Database (http://www.autophagy.lu/index.html) (Supplementary Table 2).

2.2. Determination the Alteration of OSRGs during the Coculture Process between NPCs and MSCs. The single sample gene set enrichment analysis (ssGSEA) is a bioinformatic approach to determine that whether a priori defined set of genes has statistical significance and concordant differences between two biological conditions for a single sample [13]. To investigate the alteration of OSRGs during the coculture process between NPCs and MSCs, the ssGSEA algorithm was applied to calculate the oxidative stress score of each cell sample [28]. The oxidative stress score was compared between NPC/MSC samples and NPC sample.

2.3. Identification of Differentially Expressed Genes (DEGs), Oxidative Stress-Related DEGs (OSRDEGs), and Differentially Expressed lncRNAs (DElncRNAs). Both DEGs and DElncRNAs were obtained from the GSE112216 with the criterion of adjust $p < 0.05$ and fold change > 1.50. The OSRDEGs were obtained with the intersection of DEGs and OSRGs using the Venn diagram. Volcano plots and heat maps were generated using the R package ggplot2.

2.4. Functional Enrichment Analysis and Protein-Protein Interaction (PPI) Analysis of OSRDEGs. Gene ontology (GO) analysis was conducted to explore the enriched biological process, cell component, and molecular function of OSRDEGs. Besides, the related signaling pathways of OSRDEGs were determined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The GO and KEGG functional enrichment analyses were performed using the DAVID database (https://david.ncifcrf.gov/) [29]. GO and KEGG items with $p < 0.05$ were considered as significantly enriched, and some of significantly enrich items were visualized using the R package ggplot2. The PPI analysis was conducted using the STRING database (https://cn.string-db.org/), and protein pairs with score > 0.40 were further used to build the PPI network using the Cytoscape software (https://cytoscape.org/). The PPI score was calculated using the Degree method in the cytoHubba plug-in, and top 10 OSRDEGs ranked by the PPI score were considered as the hub OSRDEGs.

2.5. Construction of DElncRNA-miRNA-Hub OSRDEG Regulatory Network. The correlation analysis between DElncRNAs and hub OSRDEGs was performed, and

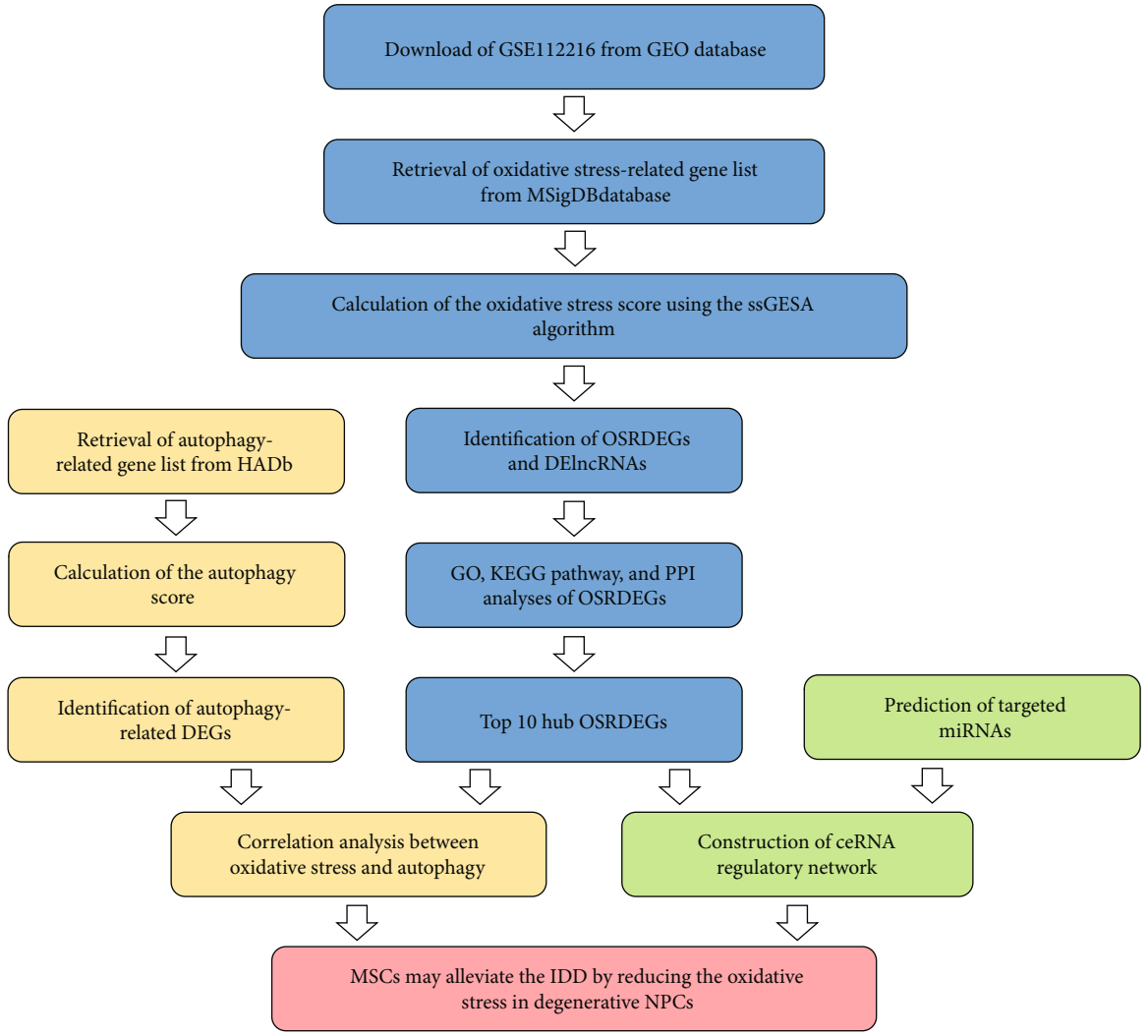


Figure 1: Flow chart of the bioinformatic analysis in the study.

DElncRNA-OSRDEG pairs with $r > 0.95$ and $p < 0.05$ were selected. The targeted miRNAs for 10 hub OSRDEGs were predicted using TargetScan database (http://www.targetscan.org/vert_80/) [30]. The targeted miRNAs for DElncRNAs were predicted using the ENCORI database (https://starbase.sysu.edu.cn/) [28]. Ultimately, the DElncRNA-miRNA-hub OSRDEG regulatory network was constructed using the Cytoscape software.

2.6. Correlation Analysis between Oxidative Stress and Autophagy. To further explore the potential role of autophagy during the coculture process, the autophagy score for each cell sample was calculated using the ssGSEA algorithm [5], and compared between NPC/MSC samples and NPC samples. To obtain the autophagy-related DEGs, the intersection between autophagy-related genes and DEGs was conducted using the Venn diagram. To detect the potential relationship between oxidative stress and autophagy in degenerative NPCs cocultured with MSCs, the correlation analysis between oxidative stress score and autophagy score was conducted. Furthermore, the relationship between hub OSRDEGs and autophagy-related DEGs was explored using the correlation analysis.

2.7. Statistical Analysis. All statistical analyses were performed using the R software 4.1.2. The ssGSEA score for oxidative stress and autophagy between NPC/MSC samples and NPC samples were compared using the Student's t-test, and $p < 0.05$ indicated there was a significant difference between NPC/MSC samples and NPC samples. Correlation analysis was conducted using the Pearson test. All p values were two sides, and p value less than 0.05 indicated there was a significant difference.

3. Results

3.1. MSCs Might Alleviate the Oxidative Stress in Degenerative NPCs. As shown in Figure 2(a), according to the preset criterion (fold change > 1.5, $p < 0.05$), a total of 106 DEGs were determined, and the clustering analysis showed these DEGs could clearly distinguish the NPC/

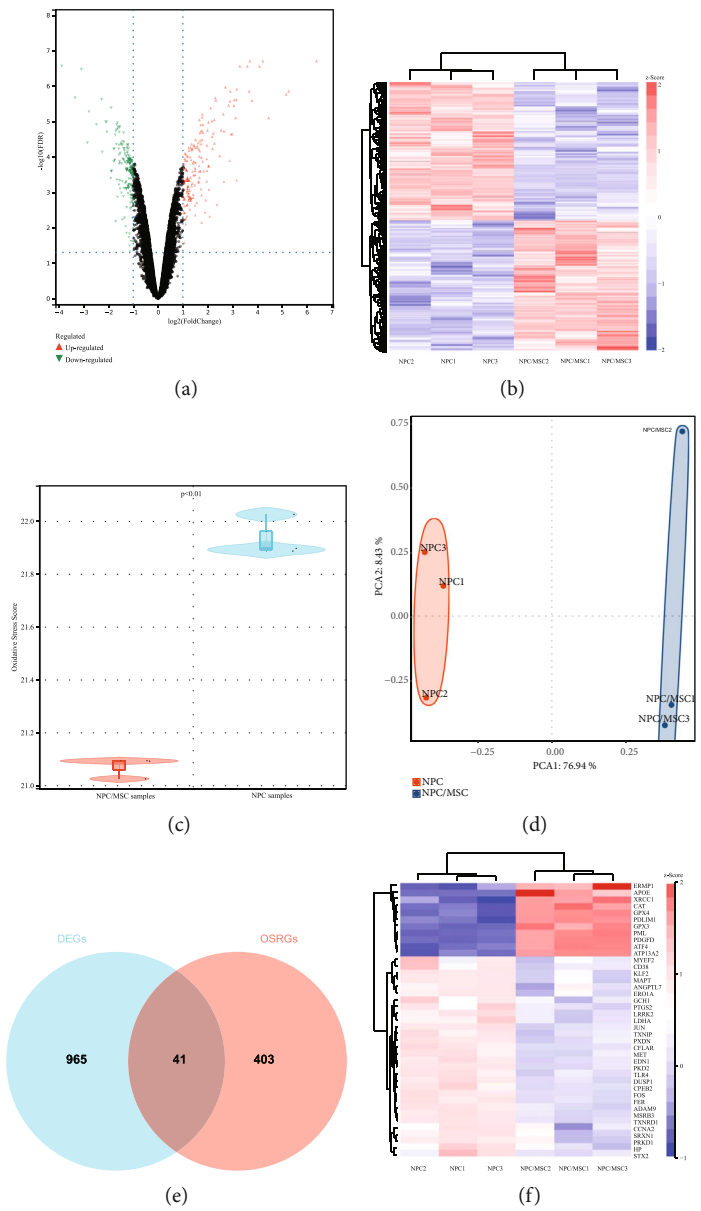


Figure 2: Determination of OSRDEGs in degenerative NPCs. (a) Volcano plot of DEGs; (b) Heat map of DEGs; (c) Comparison of oxidative stress score between NPC/MSC samples and NPC samples; (d) Principal component analysis of OSRGs; (e) Venn diagram to obtain the OSRDEGs; (f) Heat map of OSRDEGs.

MSC samples and NPC samples (Figure 2(b)). Oxidative stress is an important contributor to the IDD [6, 13]. To explore whether MSCs changed the oxidative stress status of degenerative NPCs, the oxidative stress score for each cell sample was calculated. There was a significant difference between NPC/MSC samples and NPC samples in terms of oxidative stress score (Figure 2(c)). The principal component analysis showed OSRGs could clearly distinguish the NPC/MSC samples and NPC samples (Figure 2(d)), which indicated that stem cell therapy might treat the IDD via relieving the oxidative stress in NPCs. To further investigate the underlying mechanisms, forty-one OSRDEGs were obtained by intersecting the DEGs with OSRGs (Figure 2(e)), and 11 of them were upregulated and 30 of them were downregulated (Table 1). As shown in the heat map (Figure 2(f)), the OSRDEGs significantly differed between the NPC/MSC samples and NPC samples.

3.2. Function Enrichment Analysis and PPI Analysis of OSRDEGs. The identified OSRDEGs were mapped into the GO term and KEGG pathway enrichment analyses. As shown in Figure 3(a), the following biological processes were significantly affected: Response to oxidative stress, positive regulation of transcription and DNA-templated, positive regulation of transcription from RNA polymerase II promoter, cellular oxidant detoxification, and so on. The most enriched cellular component terms were Cytoplasm, Nucleus, Cytosol, Extracellular exosome, and so on

Table 1: Detailed information of 41 OSRDEGs.

Expression	Gene symbols	Number (n)
Upregulated	ERMP1, CAT, ATF4, GPX3, PML, GPX4, XRCC1, APOE, ATP13A2, PDLIM1, and PDGFD	11
Downregulated	EDN1, FOS, PKD2, KLF2, DUSP1, MET, GCH1, PTGS2, LRRK2, JUN, LDHA, TXNIP, FER, MSRB3, TLR4, CFLAR, MYEF2, CD38, ADAM9, CPEB2, SRXN1, MAPT, PXDN, PRKD1, HP, TXNRD1, ANGPTL7, CCNA2, STX2, and ERO1A	30

OSRDEGs, oxidative stress related differentially expressed genes.

(Figure 3(b)). The most enriched molecular function terms included Identical protein binding, Peroxidase activity, Antioxidant activity, Protein homodimerization activity, and so on (Figure 3(c)). With respect to the KEGG pathway enrichment analysis, the following pathways were most affected: TNF signaling pathway, MAPK signaling pathway, Reactive oxygen species, Apoptosis, IL-17 signaling pathway, and so on (Figure 3(d)).

The PPI analysis was conducted using the STRING database and visualized using the Cytoscape software (Figure 4(a)). Top 10 hub genes were obtained according to the PPI score, including JUN, CAT, PTGS2, TLR4, FOS, APOE, EDN1, TXNRD1, LRRK2, and KLF2 (Figure 4(b)). As listed in Table 2, 8 hub OSRDEGs were downregulated and 2 hub OSRDEGs were upregulated in NPC/MSC samples when compared to NPC samples. Moreover, the correlation analysis among these 10 hub OSRDEGs was conducted, and 45 significantly related pairs ($|r| > 0.90$, $p < 0.05$) were observed (Figure 4(c)). JUN-EDN1 was the most positively related pair ($r = 0.99$, $p < 0.01$) (Figure 4(d)), and CAT-TXNRD1 was the most negatively related pair ($r = -0.99$, $p < 0.05$) (Figure 4(e)).

3.3. Construction of DElncRNA-miRNA-Hub OSRDEGs Regulatory Network. LncRNAs can exert the important biological functions as the miRNA sponges in the ceRNA regulatory network [29, 31]. A total of 27 DElncRNAs were determined (fold change > 1.50, $p < 0.05$) (Figure 5(a)), and the heat map showed these DElncRNAs could obviously distinguish the NPC/MSC samples and NPC samples (Figure 5(b)). To construct the ceRNA regulatory work, the correlation analysis between DElncRNAs and hub OSRDEGs was conducted, and DElncRNA-OSRDEG pairs with $r > 0.95$ and $p < 0.05$ were selected to construct the ceRNA regulatory network (Figure 5(c)). The targeted miRNAs for DElncRNA-OSRDEG pairs were predicted using the ENCORI database and TargetScan database. Ultimately, a total of 17 DElncRNAs, 240 miRNAs, and 10 hub OSRDEGs were applied to construct the ceRNA regulatory network (Figure 5(d)) (Supplementary Table 3).

3.4. Relationship between Hub OSRDEGs and Autophagy-Related DEGs. Previous studies have shown that autophagy played a protective role against the oxidative stress in degenerative NPCs [24, 30, 32]. In this research, a significantly different autophagy score between NPC/MSC samples and NPC samples was observed (Figure 6(a)). And there was an obvious association between oxidative stress score and autophagy score (Figure 6(b)), which indicated that MSCs might resist again the oxidative stress through restoring the autophagy in degenerative NPCs. To further explore the underlying mechanisms, thirteen autophagy-related DEGs were obtained through the intersection between DEGs and autophagy-related genes (Figure 6(c)), and the cluster analysis showed these autophagy-related DEGs could distinctly distinguish the NPC/MSC samples and NPC samples (Figure 6(d)). The correlation analysis between hub OSRDEGs and autophagy-related DEGs was conducted, and 125 significantly related pairs were obtained ($|r| > 0.90$, $p < 0.05$) (Figure 6(e)). GABARAP-CAT was the most positively related pair ($r = 0.99$, $p < 0.01$) (Figure 6(f)) and GABARAP-TXNRD1 was the most negatively related pair ($r = -0.99$, $p < 0.01$) (Figure 6(g)).

4. Discussion

IDD has become the principal contributor to the LBP, which heavily affects the life quality of patients and brings a huge economic burden to the society [2, 5]. Stem cell therapy has been considered as a promising therapeutic option for IDD, however, the involved underlying mechanisms remain unclear to date [33–36]. In the current study, we used a series of strict bioinformatic algorithms based on the sequencing data to determine the potential mechanisms involved in the stem cell therapy for IDD. We observed a significantly different oxidative stress score between NPC/MSC samples and NPC samples, which indicated that MSCs might alleviate the IDD via suppressing the oxidative stress in degenerative NPCs. Then, we determine the OSRDEGs, and explored the potential biological process and signaling pathways relevant to these OSRDEGs. Moreover, we got 10 hub OSRDEGs most worthwhile further exploring, and constructed the ceRNA regulatory network. More importantly, we found that autophagy might play an important role in the process of MSCs relieving the oxidative stress in degenerative NPCs. To the best knowledge of us, this study was the first bioinformatic analysis to investigate the possible mechanisms involved in the stem cell therapy for IDD.

Oxidative stress has been demonstrated to play a key role in the pathogenesis of IDD [6, 13, 16]. Oxidative stress could induce the apoptosis of normal NPCs, destroy the matrix proteins, and thus damage the mechanical characteristics of IVDs [16]. Some studies have explored the potential role of oxidative stress in the stem cell therapy for IDD [33, 37] . Hu et al. study showed that bone MSCs could alleviate the compression-induced apoptosis of NPCs through inhibiting the oxidative stress via the exosomes [37]. Similarly, Chen et al. reported that bone MSCs could relieve the

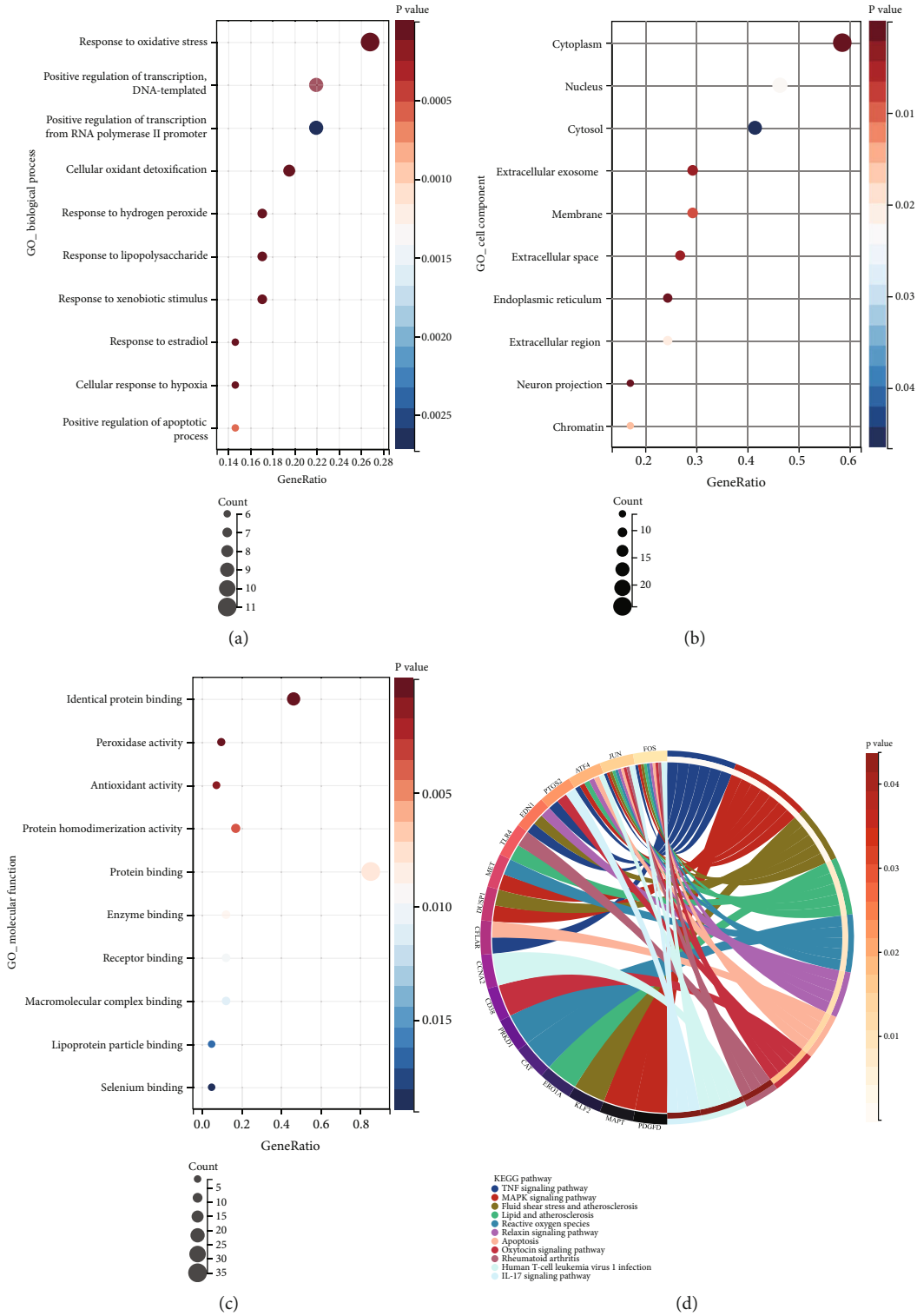


Figure 3: Functional enrichment analysis of OSRDEGs. (a) GO_ biological process; (b) GO _ cell component; (c) GO_ molecular function; (d) KEGG analysis.

compression-induced mitochondrial damage of NPCs through reducing the reactive oxygen species level and maintaining the mitochondrial functions [33]. In the current study, we observed a significantly different oxidative stress score between NPC/MSC samples and NPC samples, which indicated that stem cell therapy might improve the IDD through alleviating the oxidative stress in degenerative NPCs.

To further explore the potential underlying mechanisms involved in the stem cell therapy for IDD, we obtained 41 OSRDEGs and explored their main biological functions. The most enriched biological process was Response to

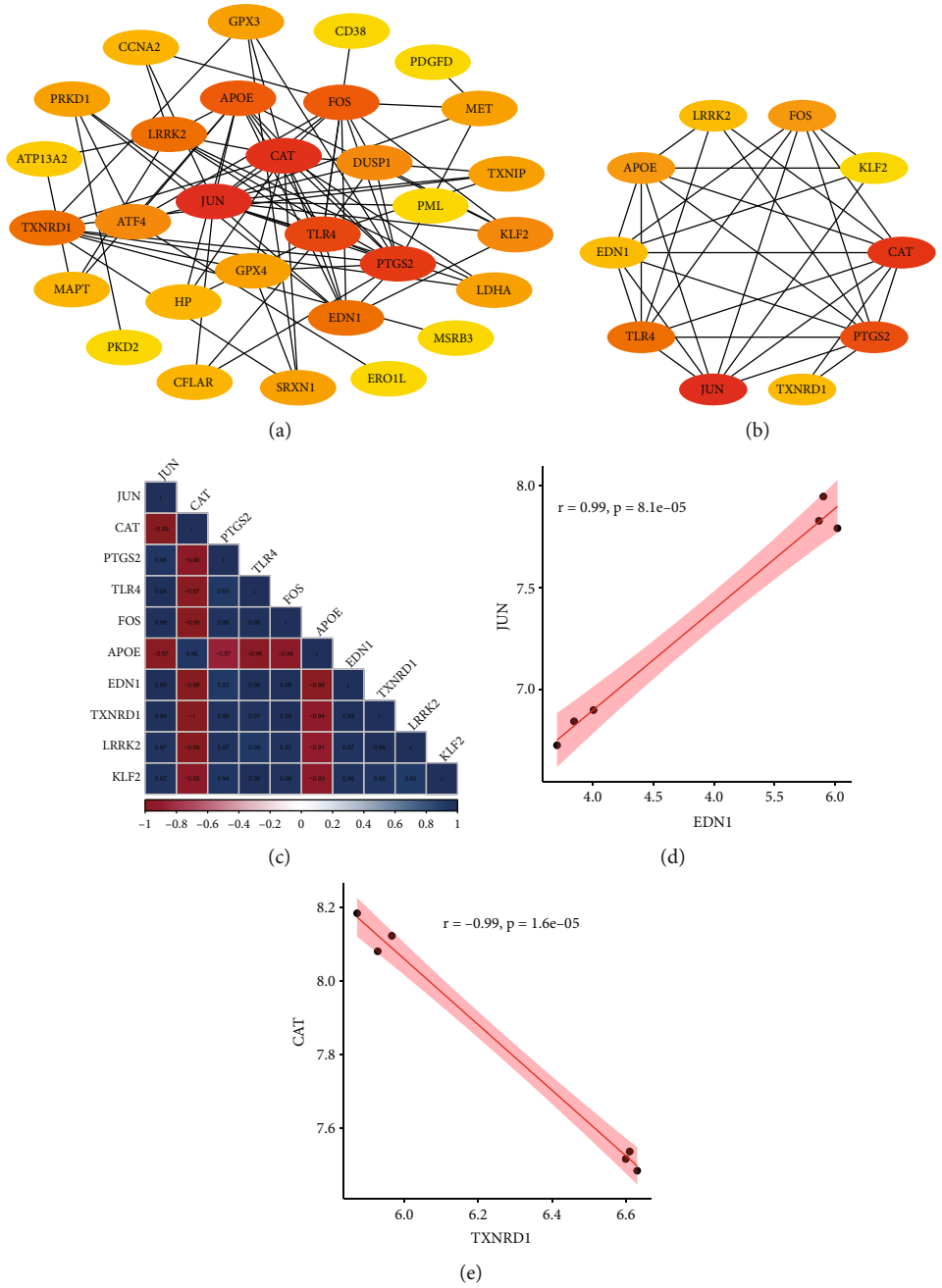


Figure 4: PPI analysis of OSRDEGs. (a) PPI analysis; (b) Top 10 hub OSRDEGs; (c) Correlation analysis among 10 hub OSRDEGs; (d) Correlation analysis between JUN and EDN1; (e) Correlation analysis between CAT and TXNRD1.

oxidative stress, cellular component was Cytoplasm, and molecular function was Identical protein binding. More importantly, we also investigated the potential signaling pathways involved in the repair process of NPCs cocultured with MSCs, and some of these signaling pathways have been proved to play important roles in the pathophysiology of IDD [38–42]. TNF signaling pathway and IL-17 signaling pathway both were inflammation-related pathways, which indicated that MSCs might reduce the oxidative stress, and then improve the inflammatory status of degenerative NPCs [38, 39]. MAPK signaling pathway was another vital biological pathway in the development of IDD [40–42]. Zhang et al. reported that platelet-derived growth factor-BB could prevent the IDD through activating the MAPK signaling pathway [40]. Cui et al. study showed that microRNA-129-5p could alleviate the IDD via blocking the LRG1-mediated p38 MAPK activation [41]. Sun et al. research indicated that calcitonin gene-related peptide could regulate the apoptosis and inflammation of NPCs via the MAPK signaling pathway during the IDD [42]. For the first time, we discovered that Relaxin signaling pathway and Oxytocin signaling pathway might exert vital functions in the biological remediation of

Table 2: Detailed information of top 10 hub OSRDEGs.

Gene symbols	Full names	Gene function	Log2 (fold change)	*P* value	Regulation
JUN	Jun proto-oncogene	This gene encodes a protein which can regulate the gene expression via interacting directly with specific target DNA sequences.	-1.03	<0.01	Down
CAT	Catalase	This gene encodes the catalase, which is an important antioxidant enzyme in the bodies against the oxidative stress.	0.62	<0.01	Up
PTGS2	Prostaglandin-endoperoxide synthase 2	The protein encoded by this gene is a vital enzyme in the process of prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase.	-1.12	<0.01	Down
TLR4	Toll like receptor 4	The protein encoded by this gene is a member of the toll-like receptor family, which is involved in the pathogen recognition and activation of inherent immunity.	-0.88	<0.01	Down
FOS	Fos proto-oncogene	This gene encodes one member of leucine zipper proteins that can dimerize with proteins of the JUN family to form the transcription factor complex AP-1.	-1.63	<0.01	Down
APOE	Apolipoprotein E	The protein encoded by this gene is a major apoprotein of the chylomicron, which is indispensable for the catabolism of triglyceride-rich lipoprotein constituents.	1.19	<0.01	Up
EDN1	Endothelin 1	This gene encodes a preproprotein that is proteolytically processed to produce a secreted peptide. This gene is involved with the tumorigenesis and pulmonary arterial hypertension.	-2.08	<0.01	Down
TXNRD1	Thioredoxin reductase 1	The protein encoded by this gene is a member of the pyridine nucleotide-disulfide oxidoreductase family, and the thioredoxin system.	-0.69	<0.01	Down
LRRK2	Leucine rich repeat kinase 2	This gene is a member of the leucine-rich repeat kinase family, and the dysregulated expression of this gene may lead to the Parkinson disease-8.	-1.09	<0.01	Down
KLF2	Kruppel like factor 2	The protein encoded by this gene is a member of Kruppel family of transcription factors. It plays an important role in the adipogenesis, embryonic erythropoiesis, epithelial integrity, inflammation and t-cell viability.	-1.29	<0.01	Down

OSRDEGs, oxidative stress-related differentially expressed genes.

degenerative NPCs cocultured with MSCs. Both relaxin and oxytocin have been demonstrated to exert important protective effects in human diseases by inhibiting the cell apoptosis [43–47]. Therefore, we speculate that MSCs may relieve the oxidative stress by activing the Relaxin or Oxytocin signaling pathways, and then prevent the apoptosis of NPCs, which is very worthy of further investigation.

Through a series of bioinformatic methods, 10 hub OSRDEGs were selected, including JUN, CAT, PTGS2, TLR4, FOS, APOE, EDN1, TXNRD1, LRRK2, and KLF2. PTGS2 was upregulated in degenerative NPCs, and associated with the inflammation in IDD [48]. TLR4 inhibition could reduce the LBP, pain-related neuroplasticity, and inflammation of disc in mice [49]. Knockout of APOE could accumulate the selective inflammatory catabolic factors, which aggravated the imbalances between catabolic and anabolic factors and deteriorated the premature IDD [50]. LRRK2 contributed to the pathogenesis of IDD, and knockdown of LRRK2 could inhibit the oxidative stress induced apoptosis through the mitophagy [51]. The potential roles of JUN, CAT, FOS, EDN1, TXNRD1, and KLF2 in IDD have not been investigated in details up to now, and deserve the further investigation. Plenty of studies have shown that lncRNAs could sponge miRNAs, also named as ceRNA regulatory network, to regulate the gene expression at a posttranscriptional level [52, 53]. To further explore the potential underlying mechanisms associated with hub OSRDEGs, we constructed the DElncRNA-miRNA- hub OSRDEG regulatory network containing 17 DElncRNAs, 240 miRNAs, and 10 hub OSRDEGs, which should be further studied in the future.

Autophagy is a catabolic process that recycles the cellular components and damaged organelles caused by various stress status [16, 54]. The autophagy level was higher in degenerative NPCs compared with normal NPCs, which indicated that autophagy might be involved in the deterioration of IDD [55]. Many investigations have indicated that autophagy was an important protective factor for IVD, and the restoration of autophagy was a promising research direction in IDD [13, 56–58]. Some studies have indicated that MSCs could significantly increase the autophagy level, and reduce the apoptosis of NPCs [25, 59]. More importantly, there was a close relationship between oxidative stress and autophagy in IDD. Chen et al. found that the overproduction of reactive oxygen species could enhance the autophagy via the AMPK/mTOR pathway in rat NPCs [60]. Moreover, Park et al. found that high glucose-induced oxidative stress could improve the autophagy by mitochondrial damage in rat notochordal cells [61]. Chen et al. reported that H_2O_2

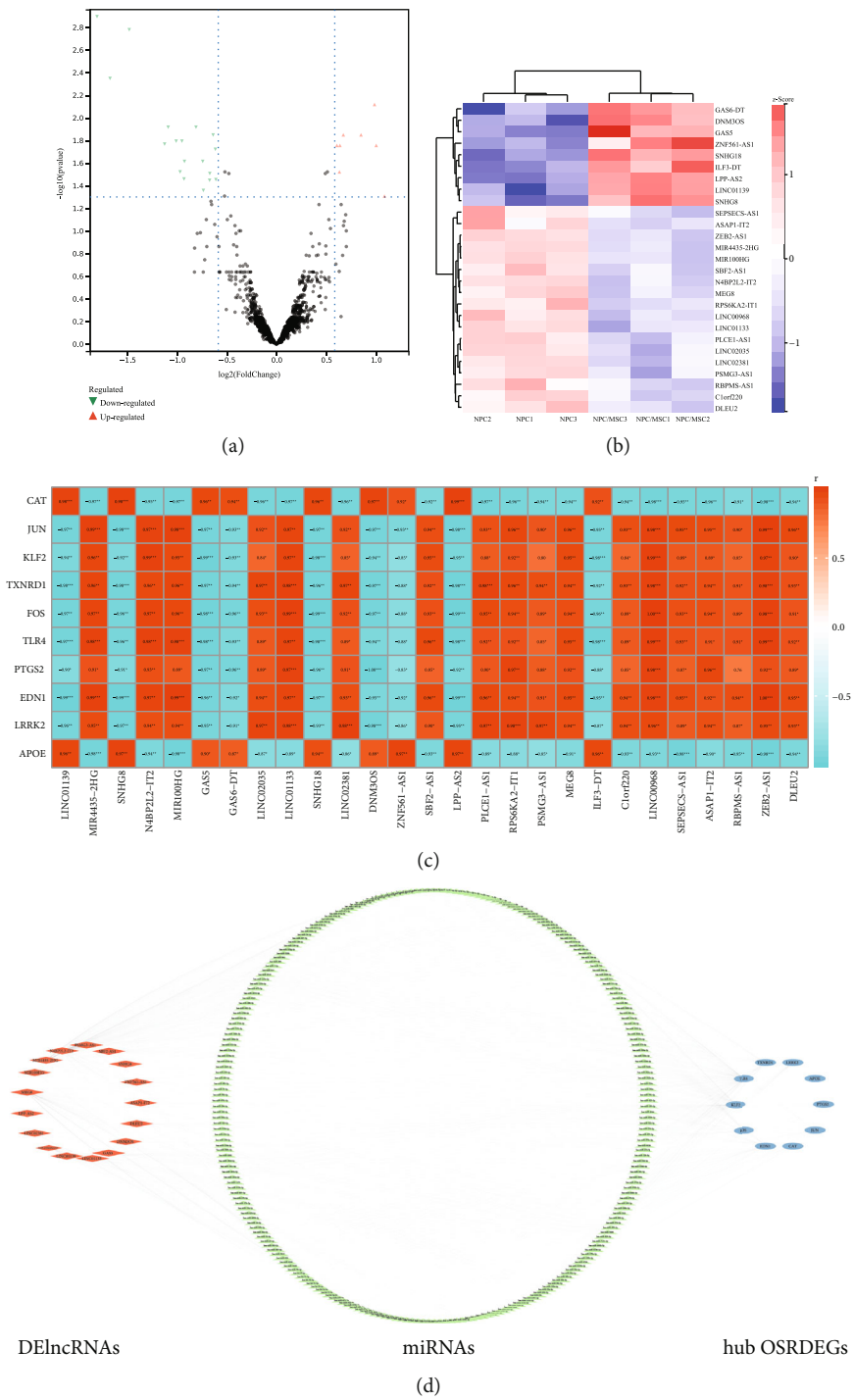


Figure 5: Construction of ceRNA regulatory network. (a) Volcano plot of DElncRNAs; (b) Heat map of DElncRNAs; (c) Correlation analysis between hub OSRDEGs and DElncRNAs; (d) The ceRNA regulatory network containing 17 DElncRNAs, 240 miRNAs, and 10 hub OSRDEGs.

could stimulate an early autophagy response through the ERK/m-TOR signaling pathway [62]. In the current study, we obtained 13 autophagy-related DEGs and performed the correlation analysis between hub OSRDEGs and autophagy-related DEGs. At last, 125 significantly related pairs were obtained, which showed that autophagy might expert vital functions in the stem cell therapy for IDD. The GABARAP-CAT pair was the most positively related pair and GABARAP-TXNRD1 pair was the most negatively related pair, and both of them should be firstly investigated in the future.

There were some limitations in the current study. First, this study was conducted based on the analysis of sequencing data. Therefore, our findings need further in vivo or vitro experiment validation. Second, oxidative stress was only one of the important pathogenic factors for IDD, and stem cell

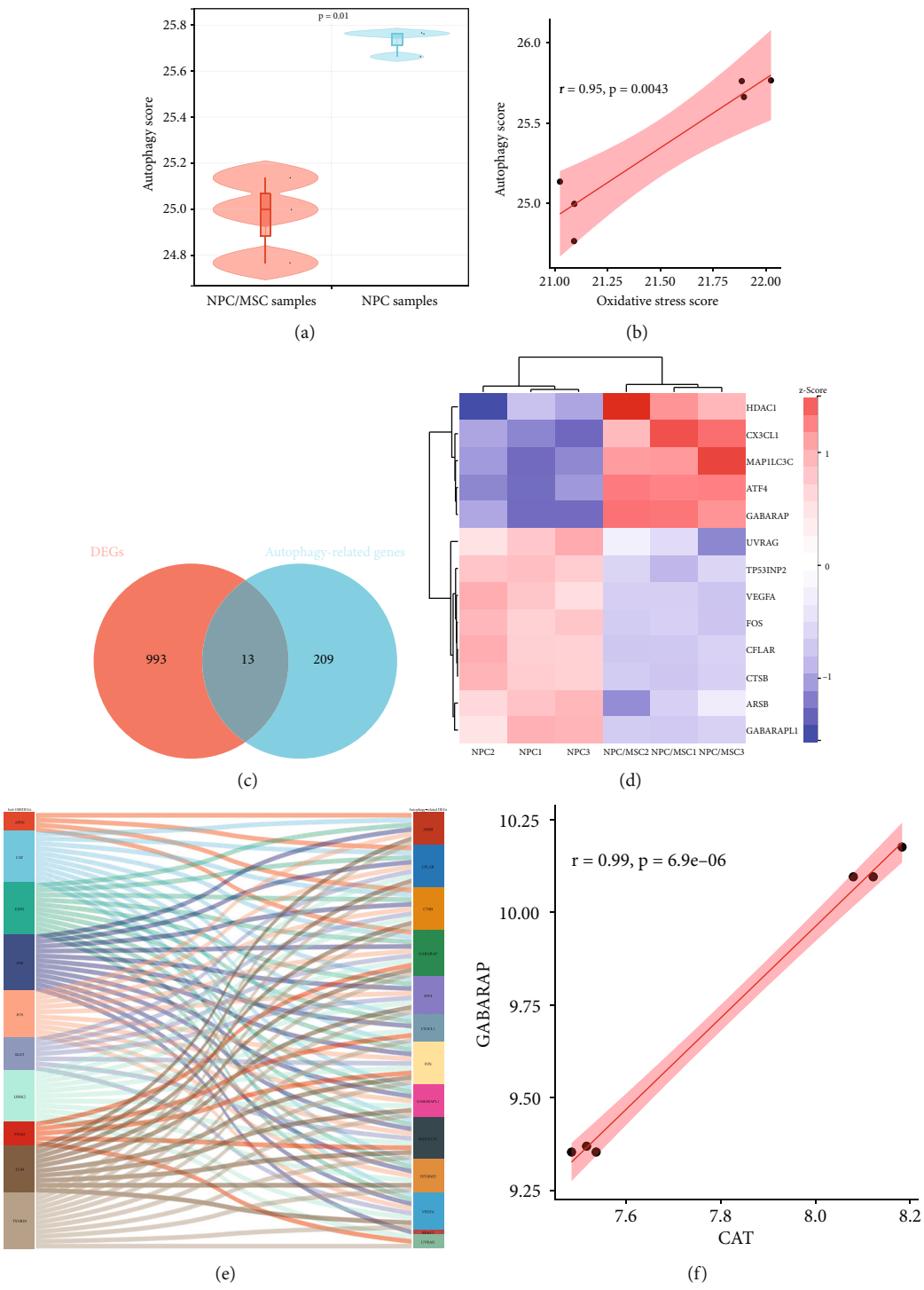
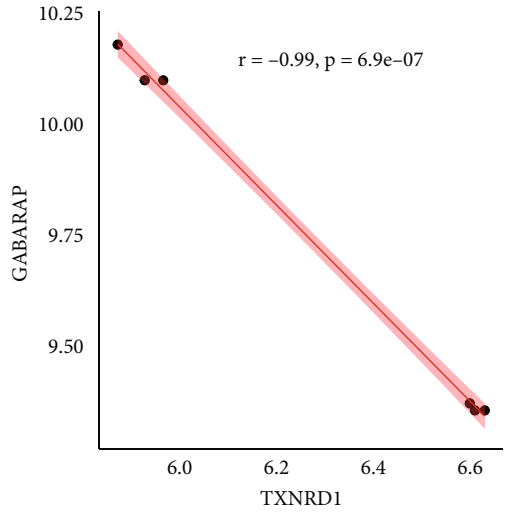


Figure 6: Continued.



(g)

Figure 6: Correlation analysis between oxidative stress and autophagy in degenerative NPCs. (a) Comparison of autophagy score between NPC/MSC samples and NPC samples; (b) Correlation analysis between oxidative stress score and autophagy score; (c) Venn diagram to obtain the autophagy-related DEGs; (d) Heat map of autophagy-related DEGs; (e) Correlation analysis between top 10 hub OSRDEGs and 13 autophagy-related DEGs; (f) Correlation analysis between GABARAP and CAT; (g) Correlation analysis between GABARAP and TXNRD1.

therapy might also repair the degenerative IVD though other pathways, such as relieving the inflammation. Third, the sequencing data used in this study was obtained from the cell samples, which could not completely simulate the degenerative IVDs treated with stem cell therapy. Forth, only six sequencing cell samples from one GEO dataset were used in this study, which might reduce the reliability of findings. Fifth, the MSCs used in this study were extracted from adipose tissues, however, there were several other sources for MSCs, such as bone marrows and embryonal tissues, which needed further investigation. Despite these limitations, the current study, for the first time, indicated that stem cell therapy might repair the degenerative IVD through resisting the oxidative stress via the ceRNA regulatory network and restoration of autophagy in degenerative NPCs.

5. Conclusion

Stem cell therapy might repair the degenerative IVD via reducing the oxidative stress through the ceRNA regulatory work and restoration of autophagy in degenerative NPCs. Further experiment studies should be conducted to validate our findings in the future.

Abbreviations

LBP: Low back pain
IDD: Intervertebral disc degeneration
MSCs: Mesenchymal stem cells
IVD: Intervertebral disc
NPCs: Nucleus pulposus cells
lncRNAs: Long noncoding RNAs
ceRNAs: Competing endogenous RNAs
miRNAs: microRNAs
GEO: Gene expression omnibus
OSRG: Oxidative stress-related gene
ssGSEA: Single sample gene set enrichment analysis
DEGs: Differentially expressed genes
OSRDEGs: Oxidative stress-related differentially expressed genes
DElncRNAs: Differentially expressed long noncoding RNAs
PPI: Protein-protein interaction
GO: Gene ontology
KEGG: Kyoto encyclopedia of genes and genomes.

Data Availability

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author/s.

Ethical Approval

This study has been approved by the Ethics Committee of Peking University Third Hospital. The written informed consent was not necessary because all data was extracted from published studies.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

Yongzhao Zhao, Qian Xiang, and Yunzhong Cheng contributed equally to this research, and were listed as the co-first authors.

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Supplementary Materials

Supplementary 1. Supplementary Table 1. The list of oxidative stress-related genes.

Supplementary 2. Supplementary Table 2 The list of autophagy-related genes.

Supplementary 3. Supplementary Table 3. Detailed information of ceRNA regulatory network.

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Review Article

Effects of Exercise or Mechanical Stimulation on Bone Development and Bone Repair

Lidan Song

Institute of Physical Education, Anyang Normal University, Anyang, 455000 Henan, China

Correspondence should be addressed to Lidan Song; songlidan@aynu.edu.cn

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The development and regeneration of the bone are tightly regulated by mechanical cues. Multiple cell types, including osteoblasts, osteocytes, osteoclasts, mesenchymal stem cells (MSCs), and recently found skeletal stem cells (SSCs), are responsible for efficient bone development and injury repair. The immune cells in the environment interact with bone cells to maintain homeostasis and facilitate bone regeneration. Investigation of the mechanism by which these cells sense and respond to mechanical signals in bone is fundamental for optimal clinical intervention in bone injury healing. We discuss the effects of exercise programs on fracture healing in animal models and human patients, which encouragingly suggest that carefully designed exercise prescriptions can improve the result of fracture healing during the remodeling phase. However, additional clinical tracing and date accumulation are still required for the pervasive application of exercise prescriptions to improve fracture healing.

1. Introduction

The skeleton senses and responds to mechanical signals while maintaining the tissue homeostasis [1]. Mechanical forces take part in regulating the process of bone development, repair, and regeneration by influencing multiple cells in the bone. As it is able to completely recover without the formation of scar, the mechanism of bone regeneration has attracted the attention of scientists and clinicians. The three phases during bone repair, the inflammatory phase, the proliferation phase and remodeling phase, involve various cell types, including neutrophils, macrophages, endothelial cells, osteoblasts, osteoclasts, mesenchymal stem cells, and skeletal stem cells [2]. We review how these components are regulated by mechanical stimulation during the repair processes. In addition to the molecular mechanism of mechanical regulation during bone repair and regeneration, in vivo studies to investigate the effects of exercise on fracture repair are introduced. The results from animal models show that mechanical stimulation during the remodeling phase significantly enhanced the formation of the callus and ultimately promoted fracture repair. We also discuss clinical research that surveyed the effects of exercise on hip fracture recovery. While some of these studies showed no difference between the exercise group and the control group, some found that patients attained better physical performance and quality of life in the exercise group. More clinical data and analysis are needed to increase the prevalence of exercise prescriptions for better recovery of fracture patients. In sum, we describe the mechanical regulation of the bone during bone development, repair, and regeneration, as well as the effects of exercise on fracture repair.

2. Homeostasic Maintenance of Bone

The bones in the body can be categorized into four types according to their shapes: long bones, short bones, flat bones, and irregular bones. Bones with specific shapes and anatomical locations function to support the posture and locomotion of the body, protect the viscera and hematopoietic system, and maintain the balance of mineral and secreted cytokines, growth factors, and other factors to execute reciprocal regulation with other parts in the body.

In long bones, the hollow shaft in the central part of the long bone is the diaphysis, where dense cortical bone dominates with the bone marrow (Figure 1(a)). In the ends of the long bones, the epiphysis above the growth plate is

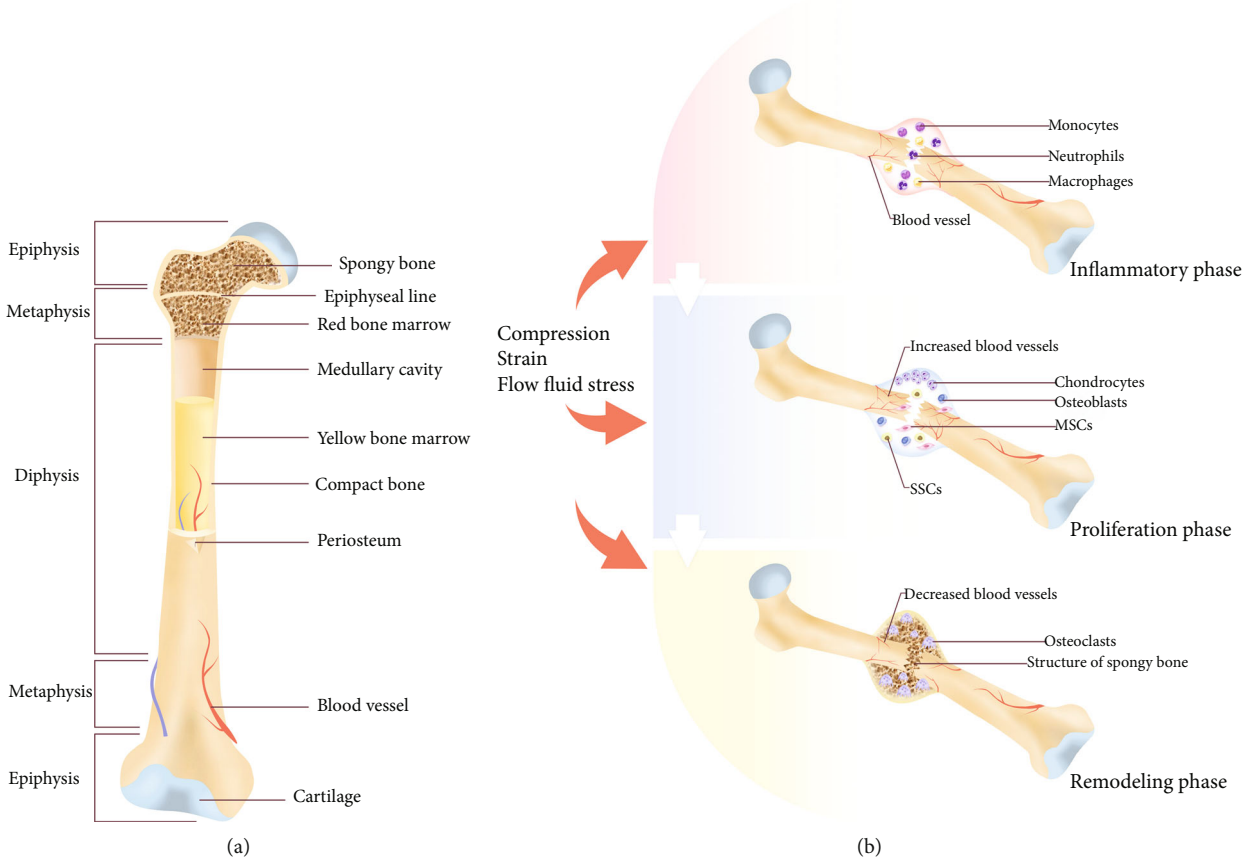

Figure 1: Bone structure and bone repair processes. (a) The basic anatomic structure of long bone. (b) The three phases in bone injury repair.

composed mainly of a trabecular meshwork bone lined by a layer of hyaline cartilage. Between the epiphysis and the diaphysis, the region below the growth plate is called the metaphysis [3]. Flat bones, such as skull and rib bones, possess a layer of sponge bone and two layers of compact bone around it.

The cortical bone is covered by periosteum and endosteum outside and inside of the bone cavity [4]. With their specific location and abundant cell types, the connective fibrous tissue, periosteum, and endosteum have been proven to participate in bone regeneration [5] and hematopoietic stem cell population preservation [6].

The maintenance of bone homeostasis is mainly dependent on the equilibrium between bone-forming osteoblasts and bone-resorbing osteoclasts. Other cell types in the skeleton include osteocytes and chondrocytes. For the maturation of osteoblasts, mesenchymal stem cells (MSCs) and skeletal stem cells (SSCs) are needed. First found in the bone marrow, bone marrow mesenchymal stem cells (BM-MSCs) are multipotent stromal cells with the ability to differentiate into osteoblasts, chondrocytes, and adipocytes. MSCs express specific markers, including CD73, CD90, and CD105 [7]. In addition to the bone marrow, MSCs can also be isolated in other sites, including the periosteum and cortical bone [8]. In the recent decade, SSCs have been found in the growth plate and periosteum, whose role in bone regeneration is discussed in the next section. The deficiency of periosteal stem cells leads to impaired postnatal skeletal growth [9]. The relationship between the two stem cell populations in the skeleton has not been clearly expounded beyond the restricted comprehension of the full cell composition. Compared with MSCs, which were found in the bone marrow [10], SSCs sorted through cell surface marker combinations are relatively newly characterized cell populations, the properties and functions of which required further research.

3. Bone Development and Tissue Repair Process

3.1. The Development of Bone. As the scaffold of the body, the development of the skeleton requires coordinated mobilization of different cells derived from multiple germ layers. Neural crest cells derived from neural ectoderm give rise to part of the craniofacial bones and cartilage in the anterior skull. The formation of the posterior skull is dependent on cells from the prechordal mesoderm. The paraxial mesoderm (somites) is responsible for the formation of the axial skeleton, while cells from the lateral plate mesoderm develop into the appendicular skeleton [11]. Two processes with different cell transitions, intramembranous ossification and endochondral ossification, mediate

ultimate bone maturation throughout the body. Intramembranous ossification is found in the development of flat bones, including the skull, mandible, maxilla, and clavicle, during which the mesenchymal cells in the condensation differentiate directly into osteoblasts and osteocytes. On the other hand, endochondral ossification involves the formation of cartilage primordium, where the mesenchymal cells in the center differentiate into chondrocytes first and the perichondrium formed by surrounding chondrocytes compartmentalizes the future bone from other surrounding tissues. The hypertrophy of the chondrocytes in the perichondrium is followed by the invasion of blood vessels, which allows for the recruitment of osteoprogenitors and cartilage-absorptive cells. Then, the bone marrow cavity, which is also called the primary ossification center and the trabecular bone and haematopoietic cells within it arise. Following the continued expansion of the primary ossification center, the secondary ossification centers, which lead to the development of the epiphysial growth plate, form in the ends of the growing bones [12]. Essential for the elongation of long bones, the growth plate is a complex region with chondrocytes at different states [13]. Located close to the epiphysis, chondrocytes in the quiescent zone serve as a pool for proliferating chondrocytes in the proliferative zone. Towards the diaphysis, chondrocytes stop proliferating to become hypertrophic. Some of these cells undergo apoptosis, while the other cells become osteoblasts [14].

3.2. Mechanical Stimulation in Bone Development. Normal development of the musculoskeletal system requires precise coordination of bone and skeletal muscle. Except for the cells that differentiate into osteolinage cells, normal development of skeletal muscle is also necessary for the occurrence of functional bones and joints. Muscle force is indispensable for correct musculoskeletal assembly. Aberrant muscle formation in paralyzed mouse embryos hinders the development of the bone by impacting the length of the growth plate and number of proliferating chondrocytes. Joint fusion in mouse embryos was found when muscle contraction was deficient [15]. During adulthood, the regulation of bone homeostasis by mechanical stimulation is more obvious. Mechanical load dynamically affects numerous aspects of bone, including the trabecular bone volume and the thickness of cortical bone [1, 16]. The discovery of the mechanosensitive channel protein Piezo1 partly explained the mechanism by which mechanical stimulation regulates bone formation.

The influence of mechanical loading on bone is not limited to bone cells. Since osteal lineage cells are niche cells of the hematopoietic system, it is rational to hypothesize that the response to mechanical loading of osteal lineage cells affects the hematopoietic cell populations. The effects of acute exercise and long-term exercise training on hematopoietic stem cell (HSC) survival, mobilization, and other characteristics before and after HSC transplantation have been discussed [17, 18]. The rapid development of single-cell RNA sequencing (scRNA-seq) technology and the refinement of cytometry allow for elucidation of the responses of the bone microenvironment, including hematopoietic cells and immune cells, to mechanical stimulation [19].

3.3. Bone Fracture Repair and Regeneration. Fracture is a frequent injury occurring in the musculoskeletal system. Some patients undergo delayed repair and nonunion, which severely impact work capability and quality of life. Investigation of the mechanism of bone repair and regeneration may provide more approaches for optimal treatment and more efficient repair. During the occurrence of fracture, the rupture of blood vessels and soft tissues directly leads to the initiation of the first phase of fracture healing, the inflammatory phase [20]. The conversion of fibrinogen into fibrin facilitates the formation of hematoma, where circulating and resident immune cells are recruited by the injury signal. Following the recruitment of neutrophils [21], macrophages invading into the injury site undergo population transition, which changes the state of the healing tissue from proinflammatory to anti-inflammatory by altering the cytokines secreted by the macrophages [22]. Precise temporal and spatial regulation of immune cell behavior, including migration and polarization, is necessary for efficient fracture repair [23]. Then, the presence of lymphocytes in the fracture site activates adaptive immunity for fracture healing [24]. In addition to immune cells, other more environmental cells have been found to regulate the process of bone regeneration. For example, Schwann cells were demonstrated to promote mandibular repair through crosstalk with skeletal stem cells [25].

After the activation and recruitment of MSCs and SSCs and the development of osteogenic progenitor cells, rapid differentiation and proliferation of osteoblasts begin the second phase, the proliferation phase. In this phase, a callus forms to turn the hematoma into a harder scaffold between the broken ends. Through endochondral ossification and intramembranous ossification, the formation of a cartilage callus by newly formed osteons completes the union of the fractured bone (Figure 1(b)).

In addition to immune cells and bone-forming and bone-absorbing cells, endothelial cells that mediate angiogenesis and vasculogenesis are indispensable in bone regeneration. The two processes of new blood formation, angiogenesis and vasculogenesis, involve the development of new blood vessels with or without a preexisting vascular component [26]. Whether both of the processes contribute to fracture healing or whether one of them dominates in the repair is still an open question. However, there is no doubt that active blood vessel formation occurs at the injury site. The blood vessels formed during fracture repair provide the hematoma or callus with oxygen, nutrients, and cells participating in the healing processes, such as MSCs. In addition, the immune cells and MSCs secrete growth factors, including vascular endothelial growth factors (VEGF), to promote the formation of new blood vessels, which also drive repair. Three isoforms of VEGF, A, B, and C, form homo- and heterodimers to regulate the cell behavior of endothelial cells by binding to their receptors, VEGFR1 and VEGFR2 [27]. The differentiation and proliferation of

endothelial cells are enhanced by VEGF, which also activates the recruitment and tube formation capacity of endothelial progenitor cells. The collapse of the intact bone and the destruction of the blood supply system result in necrosis of the perifracture tissue and hypoxia in the hematoma and adjacent tissue. By modifying the expression of hypoxia-inducible factor α (HIFα), it was been discovered that osteoblasts sense the oxygen level and couple osteogenesis and angiogenesis [28]. The expression of VEGF in osteoblasts overexpressing HIF1α was upregulated, while the long bones were dense and highly vascularized. When HIF1α was deficient in osteoblasts, a reverse phenotype of thinner and less vascularized long bones were observed. This research revealed that correctly regulated angiogenesis is crucial for bone formation and homeostasis maintenance. The fact that growth factors from osteoblasts influence the behavior of blood vessel-forming endothelial cells emphasizes the importance of cell interactions in tissue repair. When normal blood supply cannot be met, pathological cases are present [29]. Patients with abnormal distal arteriograms face a higher risk of nonunion. Impaired vascular in-growth to the callus in open fracture also increased the risk of nonunion, more tissue necrosis and reduced resistance to infection [30]. Interestingly, in the observed correlation between smoking and increased risk of fracture, it was hypothesized that smoking impedes vascularization at the fracture healing site by the action of nicotine, thus leading to delayed mineralization and unrepaired bone fracture [31]. Although public health data show that the rate of smoking in patients with tibial nonunions is higher than that in the general public [32], more evidence, including mechanistic research, is needed.

As one of the determinants of successful bone regeneration, vascularization is a target for the application of tissue engineering in bone repair improvement. The combination of VEGF and materials for bone regeneration enhancement increased blood infiltration and bone mineral density in in vivo bone defect models [33, 34].

After robust osteogenesis in the proliferation phase, the shape of the bone differs from that prior to injury, which is why osteoclasts are needed to start the remodeling phase. Recruited by receptor activator of nuclear factor-κB ligand-(RANKL-) expressing osteocytes, osteoclast precursors give rise to mature osteoclasts, which function in the resorption of the redundant bone [35]. Correct progress of these healing phases guarantees that the bone can be repaired to its uninjured form without the formation of a scar.

3.4. Stem Cells in Bone Regeneration. Given the potent multidirectional differentiation capacity of stem cells and the attractive prospects of their clinical application, the exploration of stem cells of specific tissues has not stopped since the first discovery of hematopoietic stem cells [36]. During the past decade, human and mouse skeletal stem and progenitor cell populations and their hierarchy have been identified by a combination of specific cell surface markers [37, 38]. The cell surface marker combination was determined according to the information from single-cell RNA-seq, which examined skeletal tissue cells. The differentiation potential of the populations was verified through in vitro differentiation and kidney capsule injection experiments. The definition of skeletal stem cells is not restricted to a single surface marker combination; other common molecular markers such as Ctsk [39] and Gremlin1 [40] have been found to mark a specific stem cell population. Given the diversity of the cells in bone tissue, the development of different regions is thought to be dependent on the skeletal stem/progenitor cells from corresponding locations, which has been demonstrated by evidence from lineage tracing experiments. Among the various parts, the growth plate [41] and periosteum [42] have received particular attention due to their importance for bone growth and regeneration.

Since the identification of skeletal stem cells, their participation and function in bone development, regeneration, aging, and bone-related diseases have been gradually unveiled. Gli1 was found to identify a cell population residing beneath the growth plate that produces osteoblasts during bone development and fracture repair [43]. Through the utilization of lineage tracing and cell lineage analysis, parathyroid hormone-related protein- (PTHrP-) expressing chondrocytes in the rest zone of the growth plate were identified as a population of skeletal stem cells that express skeletal stem cell surface markers and give rise to the hypertrophic chondrocytes of the growth plate [44]. The same research noted that Indian hedgehog (Ihh) signaling is involved in the preservation of this growth plate skeletal stem cell population. The SSCs identified in mice through the immunophenotype (CD45−TER119−Tie2−AlphaV +Thy−6C3−CD105+) were found to expand because of the initiation of the fracture repair process and mediate bone formation during healing [45]. The SSC population marked by the cell marker Ctsk was demonstrated to take part in fracture bone formation via intramembranous ossification [39]. Transcriptome analysis of this periosteal SSC distinguished it from other skeletal stem cell populations that mediate bone formation through endochondral ossification, which indicates the complexity and diversity of the SSC populations and their functional pathways in bones. In another study, the SSC population found in the periosteum, labeled by Mx1 and αSMA, was proven to be responsible for the generation of periosteal osteoblasts. Rapid migration of these cells to bone injury site was observed, mediated by CCL5 and its receptors CCR3 and CCR5 [46]. The discovery of the mechanism regulating the migration behavior of SSCs sheds new light on the mobilization of SSCs in bone regeneration.

Additionally, the bone marrow is a complicated environment where elaborately regulated bone cell and hematopoietic cell interactions occur. With the continuous innovation of the methods used to portray the cell populations in tissues, it is not hard to imagine that more markers will be proposed in future investigations. For instance, the invention of spatial single-cell transcriptomics, which adds spatial information to single-cell transcriptomics, significantly deepened the comprehension of tissue development and regeneration [47]. The application of this cutting-edge technology may provide new information and concepts about skeletal stem cells.

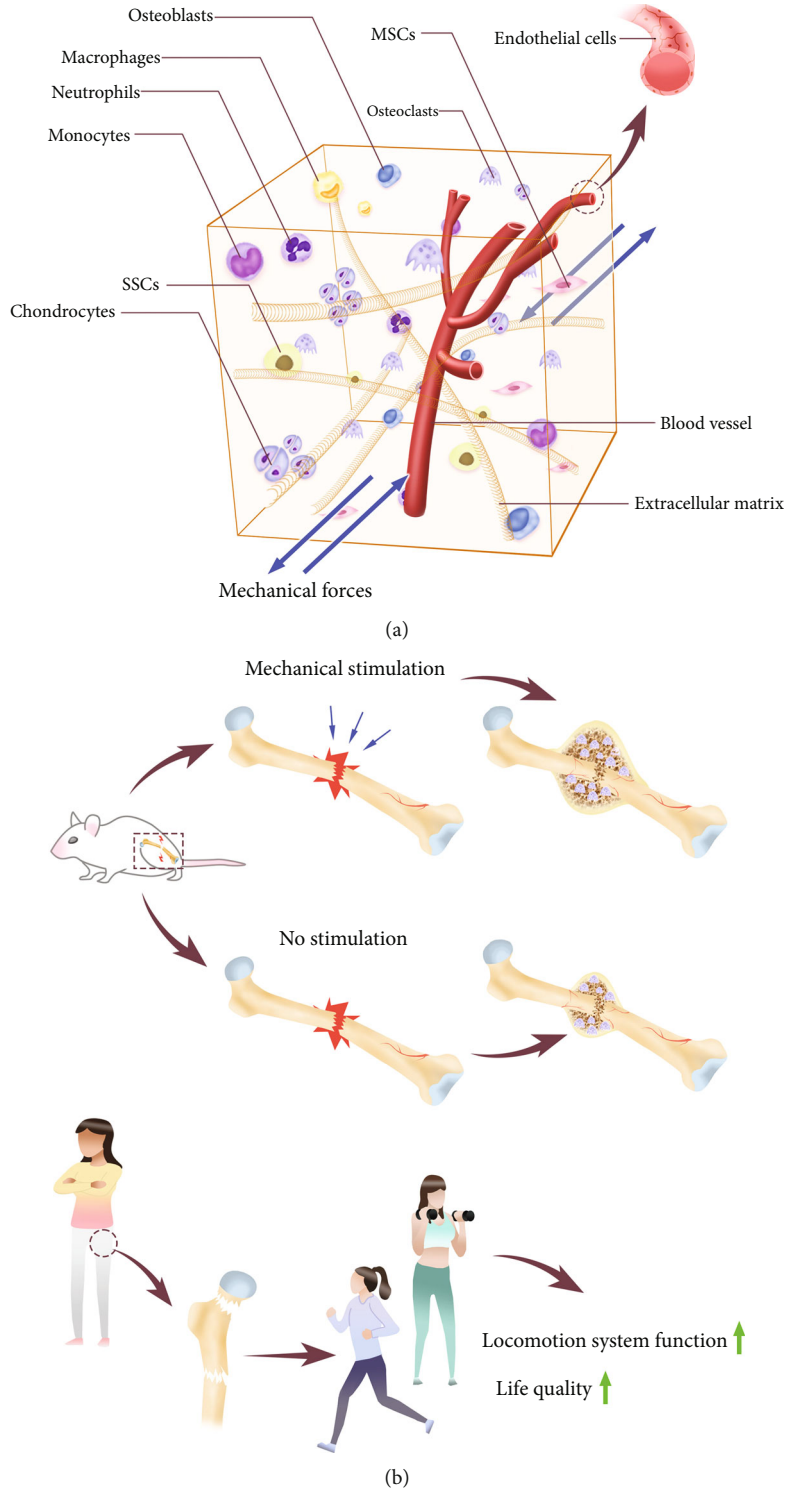

Figure 2: Mechanical stimulation and bone repair. (a) Cells and ECM in the bone tissue receive mechanical signals. (b) The effects of mechanical stimulation and exercise on bone injury repair.

4. The Role of Mechanical Stimulation in Bone Repair and Regeneration

4.1. Mechanical Stimulation in Bone Repair. In addition to the resolution of inflammation, a stable supply of the required growth factors and appropriate mechanical stimulation are necessary for bone fracture healing from the very beginning of the repair process (Figure 2(a)).

Immediately after the fracture, the fixation of the broken bone will assure normal callus formation and eventual ossification. It has been proposed that rigid fixation mainly results in intramembranous ossification, while flexible

fixation induces the process of endochondral ossification. Apparently, the mechanical strain in the fixed space affects the bone formation fashion and velocity to a large extent. During the proliferation and long-lasting remodeling phase, mechanical stimulation with proper intensity and frequency is beneficial to increase bone formation at the fracture site. Fundamentally, the influence of mechanical fixation and loading on bone regeneration is attributed to the response of the cells that function during the process and the extracellular environment and mechanotransduction between them. Through the three stages of sensation of the mechanical signal, signal transduction, and the response stage, mechanical stimulation affects the shape of the normal and injured bone. To investigate the mechanism by which the different types of cells respond to mechanical signals, various in vitro systems were used to simulate mechanical stimulation. Oscillatory fluid flow, shear stress, fluid pulse, compression, and stretch with different intensities and frequencies have been utilized to determine the response of cells and explore relevant molecules and signaling pathways.

4.2. Mechanical Regulation of the Cells in Bone. Dwelling in the lacunae, the osteocytes construct a subtle network to communicate with each other and function as mechanosensors [48]. The stress, strain, and shear fluid stress transmit mechanical signals to the osteocytes. Among the mechanical sensing proteins, Connexin43 (Cx43) has attracted significant attention as it allows for the formation of gap junctions between osteocytes and the transmission of signals, as a hemichannel protein [49]. Increased expression of Cx43 and material exchange were found in osteocytes after shear fluid stress stimulation. Structurally, it has been reported that the application of shear stress on the dendritic side of osteocytes results in the opening of hemichannels in the cells. Moreover, other mechanosensitive proteins, including transient receptor potential vanilloid 4 (TRPV4) [50] and Piezo1, have been found to mediate mechanical stimulation sensation in osteoblasts, osteocytes, and many other cells. Both TRPV4 and Piezo1 are calcium ion channels that mediate the extracellular-intracellular signal transduction through the influx of calcium ions. The Wnt/β catenin pathway and extracellular signal regulated kinase (ERK) pathway have been demonstrated to mediate the mechanical signal transduction in osteocytes [51]. In the response stage, multiple factors are produced by the stimulated osteocytes to contribute to the mechanical environment adaptation of the bone. The production of PGE2 in osteocytes, which is mediated by Cox2, accelerated bone formation [52]. However, the expression of sclerostin, an inhibitor of bone formation that antagonizes Wnt signaling, is reduced by oscillatory fluid flow stress.

Of the diverse biophysical cues that regulate the lineage commitment of mesenchymal stem cells, mechanical force is indispensable for the maintenance of bone homeostasis [53]. The conclusion that the stiffness of the extracellular matrix substrate directs mesenchymal stem cell lineage specification in cell culture provides the theoretical basis for the application of material bioengineering in tissue repair [54]. A stiffer extracellular environment induces the differentiation of osteoblasts, while a softer substrate leads to the development of adipocytes and neural cells. RhoA/ROCK signaling [55] and YAP/TAZ signaling [56] have been investigated in the mechanical regulation of mesenchymal stem cells. Although the cell population identification and regeneration participation of skeletal stem cells have been investigated during the past decade, how the mechanical stimulation regulates the cell behavior of SSCs is an important question that still requires to further investigation.

4.3. Mechanical Stimulation and Vascularization in Bone Repair. The mechanical regulation of vascularization also suggests the importance of mechanical stimulation in bone regeneration. During bone growth, active vascularization is needed. Mechanical loading of the anterior limbs of rats increased the vascularization in the periosteum [57]. A recent study reported that mechanical forces, which are associated with increased body weight at the end of adolescence, drove the differentiation of the highly angiogenic blood vessel subtype, type H vessels, into quiescent type L endothelium. The transformation of blood vessels hinders the growth of bones [58]. In a rat large bone defect model using compliant fixation plates that allow for transfer of mechanical loads or stiff fixation, early mechanical loading inhibited vascular invasion and bone formation, whereas late (after stiff fixation for 4 weeks) mechanical loading significantly stimulated vascular remodeling and bone regeneration [59]. This study highlights the mechanosensitivity of the vascular network; the evidence showed that the response of the blood vessel network to mechanical forces significantly influences bone growth and regeneration. Given the importance of vascularization in osteogenesis, researchers have tried to address the relationship between physical exercise and angiogenesis during osteogenesis. Mice and rats that underwent treadmill training had significantly larger circulating blood volumes than the sedentary control group [60]. Another study also demonstrated the adaptation of vascularization to mechanical stimulation. Rats that performed running exercise for 2 weeks had a larger number of blood vessels in the tibial proximal metaphysis and higher expression of VEGF receptor mRNA [61].

Although works investigating the effects of exercise on global vascularization have shown that exercise increases the circulating endothelial progenitor cells and angiogenic factors, specific studies on the response of angiogenesis to physical exercise during osteogenesis in humans are still lacking. New technology that allows for noninvasion monitoring of angiogenesis in patients may provide the necessary clinical data to understand how mechanical loading regulates angiogenesis and osteogenesis [62].

5. Effects of Physical Exercise on Bone Repair

Although cellular-level research is important to elucidate the molecular mechanism of mechanical regulation during bone regeneration, in vivo studies utilizing various bone injury and regeneration models are necessary to verify the proposed mechanism and assess the clinical implications of the interferences derived from mechanistic research.

Additionally, since it is not just about the individual cell behavior and cell crosstalk and interaction are involved, an in vivo study would expand our horizon for an integrative understanding of view of the bone repair and regeneration process. Recently, environmental cells, such as immune cells, endothelial cells, and pericytes, have been recognized to cooperate with tissue cells to facilitate tissue repair [63–66].

In a study using a rat fracture model, the fractured femora were mechanically stimulated 3 times a week between Day 7 and Day 18 postinjury. They found that intermittent tensile strain stimulation during fracture healing promoted chondrogenesis and had better effects on fracture repair than compressive strain or lack of stimulation, which was the control [67]. In another study using rats, approximately 6 mm defects were created in the femora. After the injury, the bones were rigidly fixed by stiff plates or compliant plates that allowed for compressive loading. Examination of the repair by microcomputed tomography, mechanical testing, and histology showed that loading significantly increased the human bone morphogenetic protein-2- (rhBMP-2-) induced regenerated bone volume [68]. Similarly, the femur defect in rats was found to be nonunion without BMP2 completed the repair efficiently. Mechanical loading enhanced the effectiveness of BMP2 in promoting bone regeneration [69]. Using time lapse in vivo imaging, this research indicated that cyclic mechanical loading significantly increased the volume of the mineralized callus of the defective bone during the remodeling phase, which is associated with the regulation of Wnt signaling [70].

The above studies assessing the effects of mechanical stimulation on fracture healing used specific animal models and surgery methods and, more importantly, customized designs for the mechanical stimulation. Different stimulation methods may lead to varying or even opposite conclusions [71]. Although mechanical stimulation realized by machine resulted in improved callus properties and healing efficiency, a study testing the effects of exercise on fracture healing in a mouse model failed to detect any significant difference between the exercise group and the control group in bone fracture with stable fixation [72]. It is possible that an adjusted exercise program or injury method would lead to different results. Therefore, it is prudent to learn the specific experimental parameters when evaluating the clinical implications of basic research. Furthermore, the surgery causing bone injury and the mechanical stimulation method require unification for more efficient and reliable communication of research achievements.

According to the encouraging results from the basic research based on animal models described above, it seems that mechanical stimulation during remodeling can be beneficial for human fracture healing (Figure 2(b)). An elaborately designed and adjusted exercise prescription can benefit patients with musculoskeletal problems. In regard to the clinical effects of exercise on bone injury repair and regeneration, the research results we can review at present are mainly concerned of the application of exercise in fracture recovery, especially for populations with impaired bone formation capacity, such as older and menopausal women. Fracture healing for most young patients is easier than for older patients because of the more exuberant bone formation capacity. Physical activity usually returns to the normal level prior to injury [73].

In the Baltimore hip study experience, women 65 years of age and older who underwent hip fracture were recruited to participate in a home-based postfracture exercise program, which included strength and aerobic components and expected the patients to exercise for 5 days per week. Although this study did not determine whether exercise improved the hip fracture healing of these frail older women, the survey showed that a home-based exercise program of strength and aerobic training after hip fracture is feasible for older patients [74]. In a randomized controlled study involving 26 older adults who experienced hip fracture, patients in the exercise group received short-term leg-strengthening exercise arranged by physical therapists, while the control group received subcutaneous electrical nerve stimulation and mental imagery. The exercise intervention was exerted twice a week for 10 weeks. Through measurements including isometric force production of lower extremity muscles, usual and fast gait speed, and a modified physical performance test etc., the study concluded that the short-term, high-intensity exercise improved the strength, walking ability, and locomotion system function of the patients compared to the control group 1 year after hip fracture [75]. A study with 33 postmenopausal women engaged in 3 months of weight-bearing and resistance training showed that exercise significantly increased the amount of osteogenic marker pro-collagen type 1 N-terminal peptide (P1NP) and circulating osteogenic cells and improved the quality of life [76]. For older hip fracture patients, a 12-month home-based exercise program intervention was also shown to improve the functioning and physical performance of the subjects compared to the patients in the control group who received the usual care only.

However, the actual situation can be more complicated than the causal relationship that physical exercise therapy improves the performance of fracture patients. There are also examinations reporting no obvious effects of physical exercise training on fracture rehabilitation. In a study that recruited 32 control and 38 intervention volunteers aged 65 years or older and had just undergone hip fracture, the intervention group received supervised high-intensity exercise training twice a week for 8 weeks. Through assessments including a one repetition maximum (1RM) test for muscle strength evaluation, a 6-minute walk test, timed up and go test, functional reach test, and observational gait analysis, they did not find significant differences between the control and intervention groups. Another randomized controlled trial recruited 124 patients who had received surgery repair of a hip fracture and gave the intervention group a twelve-month, high-intensity progressive resistance training [77]. Through the evaluation of mortality, nursing home admissions, basic and instrumental activities of daily living (ADLs), and assistive device utilization, they concluded that high-intensity weight-lifting exercise training reduced the risk of death and nursing home admissions of hip fracture patients in the intervention group. Moreover, the basic ADLs declined less and assistive device use was reduced in

the intervention group compared with the controls. In this research, exercise significantly exerted positive effects on the subjects' recovery from hip fracture. From the assessment results provided, it seems that exercise with a specific intensity that lasts for a long time can improve the quality of life of fracture patients. It is suggested that even for elderly individuals, receiving treatment for fracture, appropriate exercise training after fracture can be recommended instead of long-time inactivity. The two cases above show that it is still not feasible to directly compare the results from different clinical trials, since the exercise protocols and evaluation methods utilized can be fairly different. A study to assess the effects of weight-bearing and nonweight-bearing exercise on hip fracture rehabilitation recruited 80 inpatients who had suffered from fall-related hip fracture. The subjects were divided into two groups that received weight-bearing or nonweight-bearing exercise prescribed by a physiotherapist for 2 weeks. Strength, balance, gait, and functional performance were evaluated in the two groups. There was little difference in the improvements after receiving the two forms of exercise therapy. In this specific trial, it seems that weight bearing is not a key factor that influences the effectiveness of exercise. However, the exercise time in this case was relatively short compared with other trials that lasted for 1 year or longer. Thus, it is difficult to conclude if weight-bearing exercise lasting for a longer time would result in different outcomes.

Notwithstanding the limitations in these clinical studies in determining the effects of exercise on fracture healing, the results suggest that appropriate exercise prescriptions made by professional physical therapists can effectively improve the locomotion capability and quality of life of patients. Supervision of the exercise exertion and the tracing of the postexercise data are important to help clinicians to optimize exercise programs for fracture patients [78].

6. Conclusion

In this review, we discussed the regulation of bone development and regeneration by mechanical signals and the mechanotransduction of bone cells. As the most researched cell types, osteocytes and mesenchymal stem cells sense mechanical signals and responses and influence the balance of bone formation in healthy and pathological situations. How the mechanical response of the newly discovered skeletal stem cells influences bone regeneration is an intriguing question to explore. The molecular and cellular investigations depict the fundamental signaling pathways involved in the mechanical regulation of the bone, while the studies using animal models directly examined the effects of mechanical loading on fracture healing. The current evidence indicates that mechanical loading is positive for better callus properties and faster bone regeneration. Clinical trials involving older fracture patients showed improved healing and locomotion system function. Improved comprehension of the mechanical regulation of bone tissue and clinical data about exercise intervention influencing fracture healing are required to develop effective fracture treatment.

Conflicts of Interest

The author states that there are no conflicts of interest to report.

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Review Article

E3 Ubiquitin Ligases: Potential Therapeutic Targets for Skeletal Pathology and Degeneration

Ruiyin Zeng,[1,2] **Yuan Xiong**,[1,2] **Ze Lin**,[1,2] **Adriana C. Panayi**,[3] **Yun Sun**,[2,4] **Faqi Cao**,[1,2] **and Guohui Liu**[1,2]

[1]*Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*
[2]*Hubei Province Key Laboratory of Oral and Maxillofacial Development and Regeneration, Wuhan 430022, China*
[3]*Division of Plastic Surgery, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA*
[4]*Department of Neurosurgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*

Correspondence should be addressed to Yun Sun; 627224540@qq.com, Faqi Cao; 13971293030@163.com, and Guohui Liu; liuguohui@hust.edu.cn

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The ubiquitination-proteasome system (UPS) is crucial in regulating a variety of cellular processes including proliferation, differentiation, and survival. Ubiquitin protein ligase E3 is the most critical molecule in the UPS system. Dysregulation of the UPS system is associated with many conditions. Over the past few decades, there have been an increasing number of studies focusing on the UPS system and how it affects bone metabolism. Multiple E3 ubiquitin ligases have been found to mediate osteogenesis or osteolysis through a variety of pathways. In this review, we describe the mechanisms of UPS, especially E3 ubiquitin ligases on bone metabolism. To date, many E3 ubiquitin ligases have been found to regulate osteogenesis or osteoclast differentiation. We review the classification of these E3 enzymes and the mechanisms that influence upstream and downstream molecules and transduction pathways. Finally, this paper reviews the discovery of the relevant UPS inhibitors, drug molecules, and noncoding RNAs so far and prospects the future research and treatment.

1. Introduction

The ubiquitin-proteasome system (UPS) is comprised of several key components: ubiquitin (Ub), Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), ubiquitin ligase (E3), deubiquitinating enzyme (DUB), and proteasome. UPS is an enzymatic cascade reaction that mediates the labeling of target proteins with ubiquitin tags, leading to their degradation via the proteasome pathway. The entire ubiquitination process can be briefly described as follows: Step 1: E1 activates ubiquitin and forms an E1-ubiquitin intermediate. This process requires the consumption of ATP. Step 2: ubiquitin is transferred from E1s to E2s, forming an E2-ubiquitin intermediate. Step 3: the E3s first recognize the target protein to be degraded and then recognize the E2-ubiquitin intermediate, forming a complex containing the E2-ubiquitin intermediate, the E3s, and the target protein, and finally transfer the activated ubiquitin from E2s to the target protein. Step 4: the E2 enzyme and E3 enzyme are released from the complex, leaving the ubiquitin-tagged target protein. Step 5: the above process is repeated until multiple ubiquitin molecules are attached to the target protein to form a ubiquitin chain. Step 6: the ubiquitinated target protein is recognized and degraded into small fragments by the 26S proteasome. This process can be reversed by a group of proteases called the deubiquitinating enzymes (DUBs) which hydrolyze the peptide bond that links the target protein and ubiquitin [1].

During bone formation and reconstruction, osteogenic and osteoclastic activities need to be precisely coordinated

in order to maintain bone homeostasis. This is mainly mediated through three cell lineages: osteoblasts, osteoclasts, and osteocytes [2]. Osteoclasts differentiate from macrophages and monocytes in the human hematopoietic system and play essential roles in bone resorption [3]. Osteoblasts differentiate from mesenchymal stem cells (MSC) and synthesize, secrete, and mineralize bone matrix. Osteoblasts are the main functional cells in bone formation [4]. Osteocytes are the most common cells in mature bone tissue and are isolated from osteoblasts, which sense and transmit signals and secrete cytokines. These cells constitute the basic multicellular unit (BMU) that performs the bone reconstruction cycle [5]. Thus, the differentiation, function, and interaction of these cells are critical for regulating bone remodeling and maintaining bone homeostasis. E3 ubiquitin ligases have been found to influence osteoblasts and osteoclasts from a variety of mechanisms [6]. Therefore, regulating the relevant E3 ubiquitin ligases is an ideal approach for the treatment of the skeletal disorder.

In this review, we briefly describe the structure and function of the UPS, the mechanism of action of E3 ubiquitin ligases in bone metabolism regulation, and the E3 ubiquitin protein ligase inhibitors currently in use and molecules that are promising targets for future drug therapy.

2. Effect of E3 Ubiquitin Ligases in Skeletal Cell Fate and Pathology

E3 ubiquitin ligases can be classified into three major types based on their structures: the "really interesting new gene" (RING) family, the "homologous to E6-AP carboxyl terminus" (HECT) family, and the RING-between-RING-RING (RBR) family [7]. Different ligase domains can have specific ubiquitin transfer modes. For example, the RING E3s act as a scaffold that binds the E2 enzyme and substrate together, and ubiquitin is transferred directly from the E2s to the substrate without forming the E3-ubiquitin intermediate. However, in HECT E3s, an E3-ubiquitin intermediate is formed before ubiquitin is transferred to its substrate. More than 600 types of E3 ligases have been identified in the human genome, which contribute to the specificity of the UPS system [8].

Differentiation of the osteoblast lineage is regulated by a complex signaling pathway. Early osteoblast differentiation is mainly regulated by the BMP-SMAD-RUNX2 pathway. RUNX2 and its downstream molecule Osterix are the paramount osteoblast-specific transcription factors. This pathway triggers the expression of osteoblast phenotype genes and synthesizes bone matrix at a later stage [9]. In addition to this, Hedgehog, JNK, TGF-β, and classical Wnt/β-catenin signaling pathways are associated with the development of osteoblasts [10]. Osteoblasts then embed in the bone matrix as osteocytes or die at the end of their fate [11]. Many E3 enzymes can regulate these pathways and in turn affect osteogenesis. For example, SMURF1 acts on multiple components of the BMP-SMAD-RUNX2 and MEKK2-JNK-JUNB pathways and inhibits osteogenesis. Cdh1 regulates the MEKK2 pathway to inhibit osteogenesis. SMURF2 downregulates the TGF-β pathway, thereby hindering the PI3-kinase-AKT pathway activation, which in turn inhibits osteogenesis. WWP1 inhibits osteogenesis by promoting the degradation of SMAD4, RUNX2, and JUNB ubiquitination in osteoblasts. ITCH negatively regulates osteogenesis through JunB degradation. On the other hand, there are a number of E3 ubiquitinases that could promote osteogenesis. For example, TRIM16 reduces CHIP, therefore alleviates CHIP-mediated degradation of RUNX2, and then enhanced osteogenic. Besides, there are also proteins such as Cbl-b and c-Cbl that positively or negatively regulate bone formation by ubiquitinating the RTK-PI3K-AKT axis and other c-Cbl target proteins. In addition, insulin, through insulin-like growth factor-I (IGF-I), also affects the generation and differentiation of osteoblasts, while Cbl-b inhibits IGF-I-regulated osteogenic differentiation [12] (Table 1).

Osteoclasts are large multinucleated cells derived from the hematopoietic spectrum and regulated by several factors. Among them, the production of M-SCF and RANKL by bone marrow stromal cells and osteoblasts is essential in promoting osteoclastogenesis. M-CSF promotes the proliferation of osteoclast precursors, while RANKL stimulates the differentiation of osteoclast precursors to mature osteoclasts. In addition, the NF-κB and Wnt/β-catenin pathways also play an important role during osteoclast differentiation, which is regulated by E3 ubiquitin ligases [13]. For example, SMURF2 promotes osteoclastic differentiation by regulating RANKL expression; TRIM38 and CHIP negatively regulate NF-κB and inhibit osteoclastic differentiation; RNF146 regulates the 3BP2/SRC pathway and Wnt/β-catenin pathway and inhibits osteoclastic differentiation; LNX2 promotes activation of the NF-κB and JNK pathways and downregulation of North pathway which enhances osteoclast differentiation (Table 1).

Following, we reviewed the detailed effects of a series of E3 ubiquitin ligases which have been found to regulate the differentiation of osteoblasts and osteoclasts.

2.1. SMURF1. SMURF1, which belongs to the Hect family of E3 ubiquitin ligases, interacts with BMP pathway-specific receptor-regulated SMADs to trigger their ubiquitination and degradation, thereby inactivating them. SMADs have three subgroups: receptor-activated SMADs (for example, SMAD1, -2, -3, -5, and -8), common SMADs (for example, SMAD4), and inhibitory SMADs (for example, SMAD6 and SMAD7) [14]. SMURF1 selectively interacts with BMP pathway-targeted SMAD1 and SMAD5 to induce their degradation, thus blocking BMP-SMAD-RUNX2 signal transduction [15]. In addition, SMURF1 and SMAD (SMAD6 or 7) inhibitors synergistically negatively regulate BMP by downregulating activated BMP receptors as well as receptors of R-SMADs [16]. A regulatory circuit exists between RUNX2 and the E3 ligase SMURF1. SMURF1 acts on the C-terminal PY motif of RUNX2 and mediates RUNX2 ubiquitination, while SMAD6 enhances SMURF1-induced RUNX2 degradation [17] and RUNX2 activates SMURF1 transcription in osteoblasts [18].

TGF-β1 plays a multifaceted role in regulating osteoblast differentiation. In the early differentiation of osteoblast cells, TGF-β1 promotes proliferation and differentiation through

Table 1: E3 ubiquitin ligases and bone metabolism.

Broad group of ligase	Name	Function	References
HECT	SMURF1	Inhibits osteoblast differentiation and mineralization	[15–23, 25, 26, 28–33]
HECT	SMURF2	Inhibits osteoblast differentiation; enhances osteoclast differentiation; inhibits angiogenesis; stimulates endochondral ossification	[34–39]
HECT	Nedd4-1	Enhances osteogenic differentiation	[62–64]
HECT	Nedd4-2	Inhibits osteoblast differentiation and mineralization	[65]
HECT	WWP1	Inhibits osteoblast differentiation and mineralization	[35, 66–68]
HECT	WWP2	Enhances osteogenic differentiation	[69–71]
HECT	Itch	Inhibits or enhances osteogenic differentiation; inhibits osteoclastogenesis	[84–87]
RING	APC/C^{CDH1}	Inhibits osteoblast differentiation and mineralization	[42]
RING	APC/C^{CDC20}	Enhances osteogenic differentiation	[41]
RING	TRAF4	Enhances osteogenic differentiation	[43]
RING	TRAF6	Enhances osteoclast differentiation	[44–46]
RING	TRIM21	Inhibits osteogenic differentiation	[51]
RING	TRIM33	Protects osteoblasts against oxidative stress-induced apoptosis in osteoporosis	[52, 53]
RING	TRIM38	Enhances osteogenic differentiation; inhibits osteoclastogenesis	[54]
RING	RNF40	Enhances osteogenic differentiation	[56, 57]
RING	RNF146	Enhances osteogenic differentiation; inhibits osteoclastogenesis	[58–60]
RING	RNF185	Inhibits osteoblast differentiation and mineralization	[61]
RING	Mdm2	Enhances osteogenic differentiation	[76, 77]
RING	Cbl-b and c-Cbl	Inhibits osteoblast differentiation and mineralization; enhances osteogenic differentiation	[6, 91–95]
RING	LNX2	Enhances osteoclast differentiation	[99]
RING	Arkadia	Enhances osteogenic differentiation	[105]
B-box	TRIM16	Enhances osteogenic differentiation	[49, 50]
F-box	SCF^{Skp2}	Inhibits osteoblast differentiation and mineralization	[80]
F-box	FBL12	Inhibits osteoblast differentiation and mineralization	[96, 97]
U-box	CHIP	Inhibits osteoblast differentiation and mineralization; inhibits osteoclastogenesis	[88, 89]
RBR	Parkin	Enhances osteogenic differentiation	[102, 103]

the SMAD2/3 pathways [19]. However, TGF-β1 promotes the ubiquitination and degradation of TGF-β1 type I receptor by inducing SMURF1 and SMURF2, which in turn inhibits osteoblast mineralization during the late stages of osteoblast differentiation [20–22]. Moreover, TGF-β1 degrades the C/EBPβ protein by inducing SMURF1 expression at the transcriptional level, thereby reducing C/EBPβ-DKK1 and inhibiting matrix mineralization during osteoblast differentiation [23].

RAS-MAPK-ERK signaling pathway also plays a dual role in bone metabolism. Crosstalk exists between the TGF-β/BMP-SMAD and RAS-MAPK signaling pathways [24]. TGF-β can upregulate the expression of SMURF1 by activating the MAPK-ERK pathway, then increase the proteasome degradation of RUNX2 and SMAD1, and inhibit osteogenic differentiation [25]. Furthermore, SMURF1 can directly interact with MEKK2 and affect the activation of the downstream JNK signal cascade [26].

Tumor necrosis factor (TNF) is a proinflammatory cytokine which is one of the main factors involved in pathological bone loss [27]. One of the mechanisms of TNF in inflammatory bone disease is the induction of the expression of the ubiquitin ligases SMURF1 and SMURF2, thus promoting the ubiquitination degradation of SMAD1/5 and RUNX2 and leading to systemic bone loss [28, 29]. The possible molecular mechanism underlying is that the presence of AP-1, RUNX2, and TNF-α activates JNK and ERK, which induces JNK binding of RUNX2 and c-Jun to the SMURF1 promoter, thus promoting SMURF1 transcription [30].

Furthermore, SMURF1 can regulate cell polarity and process formation by targeting the RhoA ubiquitination degradation [31] and negatively regulating MSC proliferation and differentiation by promoting JunB degradation [32]. Continuous PTH treatment can increase SMURF1 expression in osteoblasts, leading to RUNX2 degradation and reducing anti-apoptotic signaling in osteoblasts [33] (Figure 1).

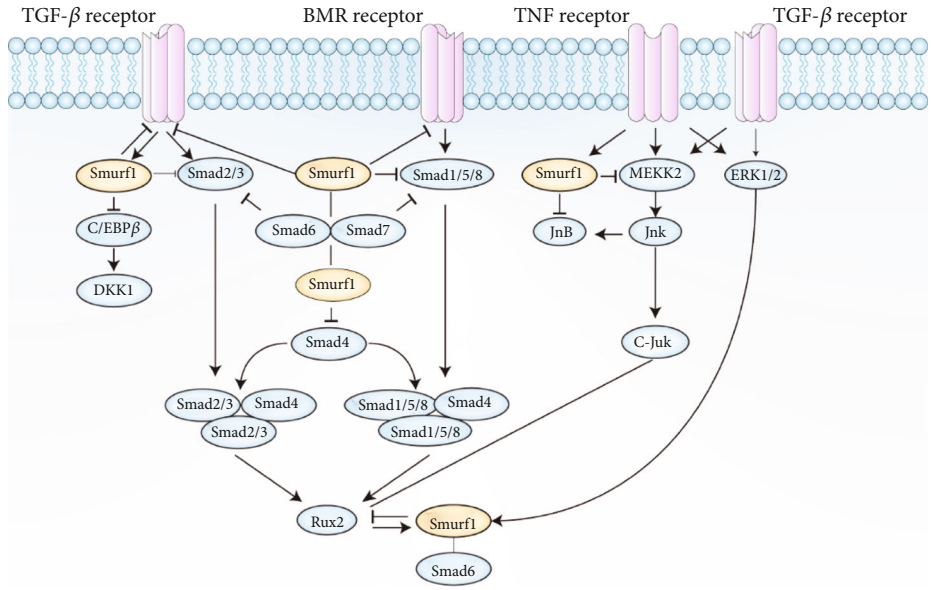

Figure 1: The E3 ubiquitin ligase SMURF1 mediates the ubiquitination and degradation of key factors from BMP/TGF-β pathway, NF-κB pathway, MAPK pathway, and other pathways, thereby regulating osteogenic differentiation. Notably, these pathways interconnected with each other and formed a complex regulatory network.

2.2. SMURF2. SMURF2 is an E3 ligase of the Hect family which mainly regulates TGF-β/BMP signaling through a pathway similar to but independent of SMURF1. SMURF2 preferentially targets SMAD1 for ubiquitination and degradation and has weaker affinity for SMAD2 and SMAD3 [34]. In addition, when SMURF2 was coexpressed with R-SMAD and SMAD2, SMURF2 showed the ability to downregulate SMAD4 similarly to SMURF1 [35]. Under IFNγ induction, SMURF2 and inhibitory SMADs (such as SMAD7) form a SMAD7-SMURF2 complex, which targets TGF-β receptors for degradation and thus bone metabolism [36]. A study showed that SMURF2 mice showed severe osteoporosis with an increased number of osteoclasts. A possible mechanism is that SMURF2-mediated SMAD3 ubiquitination affects the interaction between SMAD3 and vitamin D receptors, which regulates RANKL expression [37]. AKT is one of the key cytokines in bone anabolic signaling [12], and the PI3-kinase-AKT pathway intersects with the BMP pathway. Experiments have shown that AKT enhances RUNX2 expression by inducing SMURF2 ubiquitination and degradation which enhances the stability of the RUNX2 protein [38]. SMURF2 also stimulates chondrocyte maturation during endochondral ossification. Specifically, SMURF2 induces GSK-3 β ubiquitination and proteasome degradation, leading to the upregulation of β-catenin which promotes endochondral ossification via the Wnt signaling pathway [39].

2.3. APC/C^{CDC20} and APC/C^{Cdh1}. The anaphase-promoting complex or cyclosome (APC/C) is a multisubunit ubiquitin ligase that regulates multiple cell cycle transitions. Two APC/C activators, Cdc20 and Cdh1, directly bind to APC/C, activate its ubiquitin ligase activity, and contribute to its substrate recognition and specificity [40]. APC/C also has cell cycle-independent functions. APC/C^{CDC20} promoted the osteogenic differentiation of BMSCs by ubiquitination and degradation of p65 [41]. Conversely, the interaction between Cdh1 and SMURF1 enhances Smurf1-mediated ubiquitination of its downstream targets and inhibits osteoblast differentiation by regulating the activity of the MEKK2 pathway [42].

2.4. TRAF4. TNF receptor-associated factor 4 (TRAF4), a member of the TRAF family and a ubiquitin ligase in the RING family, plays an important role in the embryogenesis and development of the skeletal system. It was demonstrated that TRAF4 acts as an E3 ubiquitin ligase that positively regulates the osteogenesis of MSCs by mediating the ubiquitination of the K48 linkage of SMURF2 at the K119 locus and leading to its degradation [43].

2.5. TRAF6. Tumor necrosis factor receptor-associated factor 6 (TRAF6), a ubiquitin ligase in the RING family, is a key bridging molecule of the NF-κB pathway and plays an important role in the regulation of osteoclast formation. Previous studies have shown that TRAF6-deficient mice have bone abnormalities and osteosclerosis [44]. TRAF6 is essential for RANKL signaling and osteoclast differentiation. RANKL recruits TRAF6 binding to E2 ligase Ubc13/Uev1A which promotes site-specific autoubiquitination, thus activating the IKK/NF-κB and JNK/SAPK pathways which promote osteoclast differentiation [45, 46].

2.6. TRIM Family

2.6.1. TRIM16. The TRIM protein family includes about 75 proteins with E3 ligase activity and has multiple functions in proliferation, differentiation, apoptosis, carcinogenesis, and autophagy [47]. TRIM16, which belongs to the TRIM family, does not have a RING domain but has E3 ubiquitin ligase activity [48]. A study has shown that TRIM16 and

Galectin-3 coregulate the osteogenic differentiation of hBMSCs [49]. Furthermore, TRIM16 reduces CHIP, which reduces CHIP-mediated RUNX2 degradation, thus promoting osteogenic differentiation of hPDLSCs [50].

2.6.2. TRIM21. Tripartite motif containing 21 (TRIM21) is a member of the TRIM protein family with E3 ubiquitin ligase activity. TRIM21 modulated the osteogenic process of MSCs by acting as an E3 ubiquitin ligase to mediate the K48-linked ubiquitination of Akt and cause degradation [51].

2.6.3. TRIM33. Triplex protein 33 (TRIM33) is a member of the TRIM family and a RING type E3 ubiquitin ligase. TRIM33 acts as a positive regulator of osteoblast differentiation in the BMP pathway and its action is mediated by its interaction and activation with Smad1/5 [52]. In addition, TRIM33 protects osteoblasts against oxidative stress-induced apoptosis in osteoporosis by inhibiting ubiquitination and degradation of FOXO3a [53].

2.6.4. TRIM38. Triplex protein 38 (TRIM38) is a member of the TRIM family and a RING type E3 ubiquitin ligase. TRIM38 is involved in various cellular processes such as proliferation, differentiation, apoptosis, and antiviral defense. TRIM38 regulates the NF-κB pathway involved in osteoclast and osteoblast differentiation through ubiquitination and degradation of TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein 2 (TAB2) protein. Overexpression of TRIM38 in osteoclast precursor cells attenuates RANKL-induced NF-κB activation and osteoblast proliferation and differentiation. Ectopic expression of TRIM38 in osteoblast precursors negatively regulates NF-κB activation and promotes BMP2-induced IκBα phosphorylation and degradation for osteoblast differentiation [54].

2.7. RNF40. RNF40, a RING family of E3 ubiquitin ligases, monoubiquitinates histone H2A at K119 or H2B at K120, is known to function in transcriptional elongation, DNA double-strand break (DSB) repair processes, maintenance of chromatin differentiation, and exerting tumor suppressor activity [55]. A recent study has found that RNF40-driven H2B monoubiquitination is important for bone integrity in osteoblasts. RNF40 expression is essential for the early stages of lineage specification but is dispensable in mature osteoblasts [56, 57].

2.8. RNF146. RNF146 is a RING domain E3 ubiquitin ligase. Mice lacking RNF146 develop a syndrome similar to craniosynostosis dysplasia (CCD) [58]. AXIN is a key node in the Wnt pathway, and RNF146 controls the Wnt/β-linked protein pathway through ubiquitination of its substrate AXIN to inhibit osteolysis [59]. 3BP2 is the bridging protein required for the activation of SRC tyrosine kinases and coordinates the attenuation of β-linked proteins, which are necessary for osteoclast development. RNF146 also affects bone remodeling via 3BP2 ubiquitination. Furthermore, by regulating the WNT3a-FGF18-TAZ axis, RNF146 can promote osteoblast differentiation and proliferation [60]. Overall, RNF146 regulates the 3BP2/SRC and Wnt/β-catenin pathways on bone metabolism by ubiquitination of 3BP2 and AXIN1.

2.9. RNF185. RNF185, a RING type E3 ubiquitin ligase, inhibits osteogenic differentiation of mouse cranial-derived MC3T3-E1 cells. The mechanism is the interaction between RNF185 and Dvl2, a key mediator of the Wnt signaling pathway. RNF185 inhibits Wnt signaling and negatively regulates osteogenesis by promoting ubiquitin and degradation of Dvl2 [61].

2.10. NEDD4 Family

2.10.1. NEDD4-1. NEDD4/NEDD4-1, an E3 ubiquitin ligase in the NEDD4 family, is essential for osteoblast differentiation and proliferation. Lack of Nedd4 in preosteoblasts results in reduced cell proliferation and altered osteogenic differentiation. Nedd4 promotes the expansion of osteoblast progenitor cell pools which plays an important role in craniofacial development [62]. NEDD4 promotes bone formation primarily by enhancing TGF-β1 signaling. NEDD4 promotes osteoblast proliferation by degrading PTEN and TGF-β1-activated pSMAD1, upregulating pSMAD2, and promoting TGF-β1 gene expression by upregulating PERK1/2 [63, 64].

2.10.2. NEDD4-2. NEDD4-2/NEDD4L is an E3 ubiquitin ligase in the NEDD4 family. NEDD4-2/NEDD4L is similar to SMURF1 and SMURF2. Under SMAD7 participation, NEDD4-2 mediates its degradation by interacting with TβR-I. In addition, NEDD4-2 interacts with SMAD2 and induces its ubiquitinization and degradation. In general, NEDD4-2 negatively regulates the TGF-β and BMP signaling pathways [65].

2.11. WWP Family

2.11.1. WWP1. WWP1 is a member of the SMURF-like C2-WW-HECT (WW is Trp-Trp and HECT is homologous to the E6-accessory protein) type E3 ubiquitin ligases. WWP1 inhibits osteogenesis by promoting SMAD4 in osteoblasts and RUNX2 ubiquitination [35, 66]. In patients with chronic inflammatory diseases, elevated TNF inhibits bone formation through a variety of mechanisms. Junb protein is a key transcription factor that modulates MSCs to differentiate into osteoblasts. Under TNF-mediated mechanisms, WWP1 targets Junb protein proteasome degradation which inhibits bone formation [67]. In addition, WWP1 negatively regulates bone mass by inhibiting MSC migration and osteoblast differentiation. It was also found that WWP1 expression is lower in young MSCs and increases with aging [68].

2.11.2. WWP2. WWP2 is a member of the SMURF-like C2-WW-HECT type E3 ubiquitin ligases, which promotes Sox6 expression through monoubiquitination of Goosecoid under the transcriptional regulation of Sox9 and then promotes craniofacial development [69]. Besides, both WWP2 and Med25 could enhance Sox9 transcriptional activity [70]. Moreover, WWP2 promotes osteogenesis by enhancing RUNX2 through nonproteolytic monoubiquitination [71].

2.12. MDM2. MDM2 is an important negative regulator of p53 and an E3 enzyme, which promotes p53 degradation by p53 ubiquitination. P53 is an important tumor suppressor gene in the apoptosis pathway. P53, as a transcription factor, regulates cell cycle arrest, DNA repair, and apoptosis [72]. MDM2 suppresses the action of p53 on the MDM2 gene response element, thus forming a p53-MDM2 regulatory feedback loop. Therefore, in normal cells, p53 is continuously degraded through MDM2-mediated ubiquitination, resulting in a sustained low expression level of p53 [73]. Studies have shown that p53 inhibits osteoblast differentiation and osteoma formation by inhibiting the expression of RUNX2 or Osterix without affecting osteoclast differentiation [74, 75]. MDM2 negatively regulates p53 in favor of RUNX2 activation and is one of the necessary conditions for osteoblast differentiation and appropriate bone formation [76]. Dlx3 is a transcription factor that plays an important role in odontoblast differentiation. MDM2-ubiquitinated Dlx3 upregulates Dspp expression, and MDM2 ubiquitinates P53, which degrades it, reducing the inhibitory effect of mDPCs on odontoblast-like differentiation [77].

2.13. SCF^{SKP2}. SKP2 is a SCF family protein, and its complex with SKP1 and CUL1 (SCF^{SKP2}) is an E3 ubiquitin ligase [78]. This plays an important role in regulating the cell cycle [79]. SKP2 targets RUNX2 for ubiquitin-mediated degradation and thus negatively regulates osteogenesis. Moreover, RUNX2 and SKP2 expression levels in vivo are negatively related [80]. Therefore, SKP2 may be a therapeutic target for osteoporosis.

2.14. ITCH. ITCH is a HECT family E3 ligase containing the WW domain. ITCH E3 ubiquitin ligase deficiency in humans and mice leads to syndromic multisystem autoimmune disease [81]. The molecular mechanism of ITCH deficiency leading to autoimmune disease and multiorgan inflammation is related to its negative regulation of JNK and NF-κB signaling pathways [82, 83]. Therefore, the investigators found that Itch negatively regulates osteoblast differentiation from bone marrow mesenchymal stem cells through proteasome degradation of JunB protein [84]. Furthermore, Itch binds to the N-terminal part of NICD through its WW structural domain and inhibits the Notch pathway by promoting Notch ubiquitination through its HECT ubiquitin ligase structural domain [85]. Itch deficiency leads to increased expression of the Notch signal pathway and reduced differentiation of MSCs into osteoblasts, therefore resulting in osteopenic bone phenotype [86]. A study also noted an increase in osteoclasts in the bone marrow of ITCH−/− mice. One of the mechanisms is that ITCH promotes the deubiquitination of TRAF6 by recruiting CYLD to TRAF6 signal transduction complexes. TRAF6 plays an important role in RANKL signal transduction in osteoclasts and osteoclast precursors (OCP). Thus, deubiquitinated TRAF6 negatively regulates osteoclast formation via the RANKL signaling pathway [87].

2.15. CHIP. The carboxyl terminus of Hsp70 interacting protein (CHIP or STUB1) is an E3 ligase that regulates the stability of several proteins involved in different cellular functions. Deletion of the CHIP gene leads to a reduced bone mineral phenotype and increased osteoclast formation. CHIP interacts with TRAF6 to promote TRAF6 ubiquitination and proteasomal degradation, thereby inhibiting TRAF6-mediated NF-κB signaling, and plays an important role in osteoclastogenesis and bone reconstruction [88]. In addition to regulating TRAF6, CHIP inhibits TNFα-induced NF-κB signaling by promoting the degradation of TRAF2 and TRAF5 [89].

2.16. Cbl-b and c-Cbl. The Cbl (Casitas b lineage lymphoma) proteins are an evolutionarily conserved protein family that includes three different gene products (Cbl or c-Cbl; Cbl-b; and Cbl-c, Cbl-3, or Cbl-SL). Cbl-b and c-Cbl proteins are members of the mammalian CBL (Casitas B lineage lymphoma) family and are also Ring E3 ubiquitin ligases which regulate bone metabolism [90]. The effects of Cbl-b and c-Cbl on bone metabolism have been extensively studied, with the literature suggesting that Cbl proteins control osteoblast proliferation, differentiation, and survival through ubiquitination affecting the RTK-PI3K-AKT axis and other c-Cbl target proteins [6, 91, 92]. In addition, Cbl-b and c-Cbl have some less noticeable regulatory effects on bone metabolism. Osterix (also known as Sp7) is an osteogenic-specific cellular regulator which acts downstream of RUNX2 [93]. It was found that Cbl-b/C-cbl reduced the function of Osterix by degrading Osterix with ubiquitin, which inhibited bmp2-mediated osteogenic differentiation [94]. Cbl-b has been shown to be significantly increased in osteoblasts of denervated mice which inhibits IGF-I-regulated osteogenic differentiation by increasing IRS-1 ubiquitination and degradation during denervation [95].

2.17. FBL12. FBL12 is an F-box protein induced by TGF-β1. $p57^{KIP2}$ is a cyclin-dependent kinase (CDK) inhibitor (CKI) that plays an important role in cell proliferation and differentiation and affects bone development [96]. Under the stimulation of TGF-β1, FBL12 and SCF form the SCF FBL12 complex, which directly ubiquitinates $p57^{KIP2}$ and leads to its degradation, thereby inhibiting osteoblast differentiation [97].

2.18. LNX2. Notch signaling regulates proliferation, differentiation, and apoptosis in a cell-cell contact-dependent manner. It plays a crucial role in regulating the proliferation and differentiation of osteoblasts and osteoclasts in skeletal development and homeostasis in vivo [98]. LNX2 is a RING-type E3 ubiquitin ligase, which promotes the activation of ERK and AKT induced by M-CSF and the activation of NF-κB and JNK pathways stimulated by RANKL, which in turn promote osteoclast differentiation. Numb protein is an inhibitor of the Notch pathway and LNX2 binding to Numb mediates its ubiquitinated degradation and inhibits Numb-mediated inhibition of osteoblast differentiation by downregulation of the Notch pathway [99].

2.19. Parkin. Parkin (Park2) is a RING-between-RING (RBR) E3 ligase [100]. Parkin can be recruited to mitochondria and mediates mitochondrial autophagy, which is related

to the pathogenesis of Parkinson's disease [101]. It reduces ROS levels and inhibits apoptosis in osteoarthritic chondrocytes by promoting mitophagy to eliminate damaged/depolarized mitochondria [102] What is more, Parkin promotes osteoblast differentiation of BMSCs by enhancing autophagy and β-catenin signaling pathway [103]. NIPA2 is a selective Mg2+ transporter and helps maintain Mg2+ influx. NIPA2 was found to be associated with the development of type 2 diabetic osteoporosis via the mitophagy pathway. The possible mechanism underlying this is that PINK1/Parkin-mediated mitochondrial autophagy in osteoblasts is regulated by NIPA2, which is regulated by the PGC-1α/FoxO3a/MMP pathway [104].

2.20. Arkadia. Arkadia, a RING-type E3 ubiquitin ligase, is a positive regulator of the TGF-β family of SMAD-dependent signaling pathways. Arkadia promotes BMP-induced osteoblast differentiation by downregulating the BMP-specific negative regulators SMAD6, SMAD7, and c-Ski/SnoN to positively regulate BMP signaling [105].

3. UPS Inhibitors and Drugs Regulate Skeletal Cell Fate and Pathology

The most commonly used UPS inhibitors in clinical practice are proteasome inhibitors. In 2003, bortezomib (BTZ) became the first proteasome inhibitor approved by the U.S. Food and Drug Administration (FDA). BTZ has been shown to positively affect bone metabolism in MM and promote bone anabolism [106]. It directly inhibits osteoclastogenesis and promotes osteoblastogenesis [107]. Specifically, BTZ can upregulate BMP-2 expression and prevent the proteolytic degradation of the osteoblast transcription factor RUNX2/Cbfa1 to regulate osteoblast differentiation [33, 108]. BTZ inhibits osteoclast differentiation by inhibiting DKK1, RANKL, and NF-κB pathway activity [109, 110]. Experiments have shown that BTZ decreases skeletal complications of MM and prevents mechanical unloading-induced bone loss and ovariectomy-induced osteoporosis in mice [111–113].

In addition to specially developed UPS inhibitors, some commonly used drugs have also been found to be involved in bone metabolism through the UPS system, including thalidomide, lansoprazole, carnosic acid, melatonin, clomipramine, zoledronic acid, and Vitisin A. The immunomodulatory drug (IMiD) thalidomide was originally considered a teratogenic agent but is now used to treat a variety of clinical indications, including MM. It has been found that the direct target of thalidomide is the Cereblon (CRBN), a component of the cullin-4 RING E3 ligase complex. Thalidomide inhibits the ubiquitination of CRBN, leading to increased cullin-4 RING E3 ligase-mediated degradation of target proteins [114]. Recent studies indicate thalidomide has inhibitory effects on glucocorticoid-induced osteoporosis and ovariectomy-induced osteoporosis in mice, but excessive doses of thalidomide can exacerbate osteoporosis [115, 116]. Lansoprazole, which is one of the most commonly prescribed drugs for the treatment of acid-related diseases, induces TRAF6 polyubiquitination, which then activates the noncanonical TAK1–p38 MAPK pathway and facilitates Runx2-mediated osteoblastogenesis [117]. Carnosic acid (CA) is a phenolic acid compound first found in Salvia officinalis L., which possesses antioxidative and antimicrobial properties [118]. CA dually targets SREBP2 and ERRα, thus inhibiting the RANKL-induced osteoclast formation and improving OVX-induced bone loss [119]. Melatonin is a signal molecule that modulates the biological circadian rhythms of vertebrates. Melatonin treatment was found to downregulate TNFα-induced SMURF1 expression and then decrease SMURF1-mediated ubiquitination and degradation of SMAD1 protein, leading to steady bone morphogenetic protein-SMAD1 signaling activity and restoration of TNFα-impaired osteogenesis [120]. Recent studies have shown that clomipramine (CLP) induces bone loss and osteoporosis by acting on Itch to promote osteoclastogenesis. On the contrary, bisphosphonates, such as zoledronic acid (ZA) and prevent bone loss from CLP treatment [121]. One such mechanism is zoledronic acid- (ZA-) induced osteoclast cell ferroptosis by triggering FBXO9-mediated p53 ubiquitination and degradation [122]. A study found that oral administration of a drug containing (+)-Vitisin A significantly improves bone loss in ovariectomized mice. (+)-Vitisin A inhibits RANKL-induced ubiquitination of TRAF6 and formation of the TRAF6-TAK1 complex which inhibits activation of the IKK/NF-κB/c-Fos/NFATc1 signaling pathway to inhibit osteoclast differentiation [123].

There are also a considerable number of E3 ligase drugs in preclinical or clinical trials [124, 125]. The issue is that most of these inhibitors are more effective in cell culture studies and less effective in animal models and clinical trials. Therefore, further research and technological advances will be required in the future [125].

4. Noncoding RNAs Regulate Skeletal Cell Fate through the UPS System

Noncoding RNAs (ncRNAs) include intronic RNAs, microRNAs (miRNAs), long noncoding RNAs (lncRNA), circular RNAs (circRNA), and extracellular RNAs [126]. The ability of ncRNAs to control gene expression makes them viable targets for drug development. To date, several ncRNAs were found to act on E3 ubiquitin ligases to regulate bone metabolism. The lncRNA RP11-527N22.2, named osteogenic differentiation inhibitory lncRNA 1 (ODIR1), acts as a key negative regulator during the osteogenic differentiation of hUC-MSCs through the FBXO25/H2BK120ub/H3K4me3/OSX axis [127]. miR-142-5p promoted osteoblast activity and matrix mineralization by targeting the gene encoding WW-domain-containing E3 ubiquitin protein ligase 1 [128]. miR-25 secreted by BMSC-Exo regulates the ubiquitination degradation of Runx2 by SMURF1 to promote fracture healing in mice [129]. Mesenchymal stem cell-derived exosomal miR-19b represses the expression of WWP1 or Smurf2 and elevates KLF5 expression through the Wnt/β-catenin signaling pathway, thereby facilitating fracture healing [130]. BMSC-derived exosomal miR-101 augments osteogenic differentiation in MSCs by inhibiting FBXW7 to regulate the HIF1α/FOXP3 axis [131]. Silencing DCAF1 by miR-3175 activated Nrf2 signaling to inhibit dexamethasone-induced oxidative injury and apoptosis in human osteoblasts [132].

miR-764-5p positively regulates osteoblast differentiation from osteoblast progenitor cells by inhibiting CHIP protein translation [133]. In addition, biomaterials have also been used as drug delivery platforms to deliver ncRNA. In this research, regenerative siRNA against WW domain-containing E3 ubiquitin protein ligase 1 (Wwp1) complexed with hybrid nanoparticle (NP) were entrapped within poly (ethylene glycol) (PEG)-based hydrogels and implanted at sites of murine middiaphyseal femur fractures. Results showed that fractures treated with siRNA/NP hydrogels exhibited accelerated bone formation and significantly increased biomechanical strength [134].

5. Conclusions

Recognition and understanding of the role of the ubiquitin-proteasome system in osteogenic regulation have gained significance in the past decades. Its discovery has helped us understand the nature of biochemical processes behind major developmental and homeostatic events. Numerous ubiquitin enzymes have been discovered so far, with E3 ubiquitin ligases being the most important and diverse. In this review, we discuss and present the role of E3 ubiquitin ligases in bone metabolism, drawing from historical studies on E3 ubiquitin ligases in bone metabolism, as well as recent findings. They regulate bone metabolism through several key factors and pathways that act on osteogenesis and osteoclast.

Designing therapies that target each component of the UPS in order to treat pathology holds great promise for clinical practice. Some proteasome inhibitors are already in clinical use and have been shown to be effective in the treatment of multiple myeloma. Some of these drugs, such as bortezomib, were found to prevent osteoporosis in mice. The main pharmacological effects of some clinical drugs such as thalidomide, lansoprazole, carnosic acid, melatonin, clomipramine, zoledronic acid, and Vitisin A are not related to the UPS system. However, several recent studies have found that these clinical drugs could affect different E3 ubiquitin ligases, which in turn regulate different bone metabolic pathways. Noncoding RNAs, such as miRNA, lncRNA, and siRNA, have also been used to regulate bone metabolism by targeting the UPS system. However, the application of noncoding RNAs is challenged by their poor stability, poor pharmacokinetics, and potential off-target effect. The use of corresponding biomaterials will greatly improve the therapeutic efficacy of noncoding RNA. But the research in this area is relatively basic, and there is still room for further improvement. Moreover, there are a considerable number of E3 ligase drugs in preclinical or clinical trials. Further research and technological advances such as PROTAC (Proteolysis targeting chimeras) may take the research to a new level [135]. With the further discovery of the mechanisms of the E3 ubiquitin ligases related to bone metabolism, more drugs targeting E3 ligases will be designed for the treatment of skeletal disorders.

Conflicts of Interest

All authors declare no conflicts of interest.

Authors' Contributions

Ruiyin Zeng, Yuan Xiong and Ze Lin contributed equally to this work.

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Review Article

Effectiveness and Mechanisms of Low-Intensity Pulsed Ultrasound on Osseointegration of Dental Implants and Biological Functions of Bone Marrow Mesenchymal Stem Cells

Chao Liang,[1,2] **Xiu Liu**,[2] **Yuwei Yan**,[1] **Rongxin Sun**,[1] **Jun Li**,[1,2] **and Wei Geng**[1]

[1]*Department of Dental Implant Center, Beijing Stomatological Hospital, School of Stomatology, Capital Medical University, Beijing 100050, China*
[2]*Beijing Institute of Dental Research, Beijing Stomatological Hospital, School of Stomatology, Capital Medical University, Beijing 100050, China*

Correspondence should be addressed to Jun Li; lijun3021@aliyun.com and Wei Geng; gengwei717@163.com

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Dental implant restoration is the preferred choice for patients with dentition defects or edentulous patients, and obtaining stable osseointegration is the determining factor for successful implant healing. The risk of implant failure during the healing stage is still an urgent problem in clinical practice due to differences in bone quality at different implant sites and the impact of some systemic diseases on bone tissue metabolism. Low-intensity pulsed ultrasound (LIPUS) is a noninvasive physical intervention method widely recognized in the treatment of bone fracture and joint damage repair. Moreover, many studies indicated that LIPUS could effectively promote the osseointegration of dental implants and improve the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). This review is aimed at investigating the research progress on the use of LIPUS in dental implant medicine from three aspects: (1) discuss the promoting effects of LIPUS on osseointegration and peri-implant bone regeneration, (2) summarize the effects and associated mechanisms of LIPUS on the biological functions of BMSCs, and (3) introduce the application and prospects of LIPUS in the clinical work of dental implantation. Although many challenges need to be overcome in the future, LIPUS is bound to be an efficient and convenient therapeutic method to improve the dental implantation success rate and expand clinical implant indications.

1. Introduction

With the advancement of dental implant science, implant restoration has become the preferred treatment approach for patients with dentition defects or edentulous to restore oral function and aesthetics [1]. Osseointegration is an important metabolic and remodeling process involving bone tissues surrounding implant surfaces, and achieving stable osseointegration during the healing period is a prerequisite for successful dental implantation [2]. The theory of implant osseointegration was first proposed by Brånemark in 1977, who reported a direct structural and functional connection between the surface of a titanium implant and the active human bone tissues, without any connective tissue between these two components [3]. The quality of osseointegration is primarily influenced by the bone quality and bone mass at the local implant site, and systemic health factors that influence bone metabolism also play important roles [4].

Good bone quality and adequate bone mass can ensure that dental implants are placed at the ideal site, leading to a good functional and aesthetic outcome. However, periodontal disease, trauma, and bone tissue atrophy or resorption often cause alveolar bone insufficiency, and thus, different degrees of peri-implant bone defects appear after the implants are placed. Therefore, bone augmentation surgery, such as guided bone regeneration (GBR), is required to cover the exposed surface of the implant [5]. However, the period for achieving osseointegration in the bone defect region is even longer and is closely associated with the supply of peripheral blood and the migration and differentiation

of osteoblastic cells in the bone marrow [6]. At present, firmly and quickly establishing osseointegration in the bone defect area around the implant is still a clinical challenge.

Furthermore, systemic diseases, such as diabetes mellitus and osteoporosis, are considered important risk factors that impact the success rate of dental implant treatment. These diseases are usually accompanied by different degrees of bone remodeling disorders, which can interfere with the osseointegration of implants during the healing period. Although diabetes mellitus with good blood glucose control and osteoporosis under systemic medication is no longer absolute contraindications for implant surgery because of the progress in implant surface treatment technology [7], several studies have reported that the aforementioned diseases still present a potentially high risk for implant failure [8, 9]. An absence of osseointegration directly causes implant loss and surgical failure and is difficult to predict. Therefore, finding new methods to improve the osseointegration of implants simply and quickly and shortening the healing cycle have become the focus of current clinical research.

Low-intensity pulsed ultrasound (LIPUS) is an emerging noninvasive technology for physical intervention and can directly act on target tissues using pulsed ultrasound at an output intensity lower than $1\,W/cm^2$ to produce many biological effects, including promoting protein synthesis, improving cell proliferation, and increasing cellular secondary messenger calcium uptake [10, 11]. LIPUS is widely recognized as a safe and effective method for treating bone, cartilage, nerve, and soft tissue diseases and has almost no toxic or side effects to normal tissue [12–15]. Many studies have reported the promoting effect of LIPUS on tissue regeneration and cell metabolism, particularly in treating bone fracture and cartilage injury [16–18]. A systemic review and meta-analysis even defined LIPUS as the most effective method for treating bone nonunion besides surgery [19]. In addition, several studies explored the therapeutic effects of LIPUS on cartilage tissue injury of the temporomandibular joint (TMJ) in the last 5 years and found that LIPUS could effectively suppress temporomandibular joint disorders (TMDs) in rats, which was caused by chronic sleep deprivation (CSD) intervention [20–22]. A recent review article also confirmed the effect of LIPUS on osteoarthritis of the TMJ [23].

In recent years, many studies used LIPUS in the field of dental implantation, seeking to use LIPUS to improve peri-implant bone remodeling and shorten the healing cycle. *In vivo* studies showed that LIPUS could significantly increase the bone–implant contact (BIC) rate of implants and effectively promote new bone formation [24]. *In vitro* studies further confirmed that LIPUS could promote the proliferation, migration, osteogenic differentiation, and mineralization abilities of alveolar bone marrow mesenchymal stem cells (BMSCs), activate osteogenesis-associated signaling pathways, and induce BMSCs to express osteogenic cytokines and proteins [16]. LIPUS has high clinical application value in the promotion of implant osseointegration and bone regeneration around implants during the patient healing period. The present study investigated the research progress in LIPUS use in dental implant medicine from three aspects: (1) the promotive effects of LIPUS on implant osseointegration and peri-implant bone regeneration, (2) the effects and associated mechanisms of LIPUS on the biological functions of BMSCs, and (3) the application and prospects of LIPUS in the clinical work of dental implantation. Finally, future research directions have been suggested in Conclusion.

2. Promotive Effects of LIPUS on Implant Osseointegration and Peri-Implant Bone Regeneration

In the last 30 years, the treatment of bone fractures and other bone defect diseases with LIPUS has achieved landmark clinical effects. The osseointegration of implants shares many similarities with the bone fracture healing process, including blood clot filling, inflammatory response, osteoid tissue formation, and bone remodeling [25]. After implants are placed into the alveolar bone tissues, blood first fills the gap between the implant surface and the surrounding bone tissue, and then, osteoid tissues and new trabecular bone gradually replace the blood clots during the early healing stage. Next, the bone-like tissues are gradually remodeled to form lamellar bone to achieve close contact with the implant surface, and osseointegration is finally complete [26]. Many studies have verified the promoting effect of LIPUS in bone remodeling and regeneration, and in the following paragraphs, we will focus on the effects of LIPUS on implant osseointegration. Current *in vivo* studies on the application of LIPUS to dental implants are summarized in Table 1.

2.1. Interventional Effects of LIPUS on Implant Osseointegration. Many *in vivo* studies used LIPUS to promote the osseointegration of dental implants. Ustun et al. [27] showed that the intervention of dental implants in rabbit tibias for 4–6 weeks using LIPUS at $30\,mW/cm^2$ intensity significantly increased the BIC rate and the stability of implants. Liu et al. [32] used $40\,mW/cm^2$ LIPUS to treat implants in rabbit femurs and tibias, and 3 weeks of intervention significantly increased the tissue mineral density, bone volume/tissue volume (BV/TV) fraction, trabecular thickness around the implants, and pullout torque of the implants. Similarly, Zhou et al. [29] in a rat model showed that the application of LIPUS at $30\,mW/cm^2$ significantly increased the BIC rate and the BV/TV fraction in rat tibias in week 4 compared with the natural healing control group. However, the differences were not significant in weeks 8 and 12, suggesting that the advantage period of LIPUS in promoting new bone formation around implants was during the early healing period. Simultaneously, Kang et al. [28] also concluded that LIPUS could effectively promote the osseointegration of dental implants in 4 weeks in a canine model. In addition, Ruppert et al. [34] compared the effects of LIPUS and low-magnitude, high-frequency (LMHF) vibration generated by a dual-limb local vibration stimulator on implant healing in rat femurs and showed that LIPUS promoted osseointegration after 4 weeks of intervention and increased the pullout torque of implants more

Table 1: Summary of LIPUS studies on implant osseointegration and peri-implant bone regeneration.

Studies	Animal models	Titanium implants	LIPUS parameters	Time of stimulation	Major conclusions
Ustun et al. [27]	New Zealand rabbits Tibiae	Length: 6.0 mm Diameter: 4.1 mm Screw-shaped	Intensity: 30 mW/cm^2 (/SATA) Pulse frequency: 1.5 MHz	20 min/day for 1, 2, 3, 4, 5, and 6 weeks	LIPUS may have positive effects on osseointegration and stability of dental implants
Kang et al. [28]	Mongrel dogs Mandibular bone	Length: 8.5 mm Diameter: 3.3 mm	Intensity: 240 mW/cm^2 (/SATA) Pulse frequency: 3.0 MHz	15 min/day for 1 week	LIPUS may have a positive effect on osseointegration and stability of dental implants, especially in early healing periods
Zhou et al. [29]	SD rats Tibiae	Length: 4.0 mm Diameter: 2.0 mm Pitch: 0.6 mm Screw-shaped	Intensity: 30 mW/cm^2 (/SATA) Pulse frequency: 1.5 MHz	20 min/day for 4, 8, and 12 weeks	LIPUS therapy may accelerate the bone healing and osseointegration at the interlace between titanium implant and bone and promote remodeling of bone trabecula in the early stage
Nakanishi et al. [30]	Japanese white rabbits Femur	Length: 10 mm Diameter: 3.3 mm	Intensity: 40 mW/cm^2 or 100 mW/cm^2 (/SATA) Pulse frequency: 1 MHz or 3 MHz	20 min/day for 2 weeks	Clinical application of LIPUS for dental implants may promote osseointegration
Hsu et al. [31]	New Zealand rabbits Tibiae	Length: 8 mm Diameter: 3.6 mm Screw-shaped	Intensity: 50, 150, and 300 mW/cm^2 (/SATA) Pulse frequency: 1 MHz	10 min/day for 30 days	LIPUS at 0.05–0.3 W/cm^2 intensity may accelerate cell proliferation and promote the maturation of collagen fibers and support osteointegration
Liu et al. [32]	New Zealand rabbits Femur and tibiae	Length: 18 mm Diameter: 2.5 mm Screw-shaped	Intensity: 40 mW/cm^2 (/SATA) Pulse frequency: 1.5 MHz	10 min twice a day (total 20 min) for 3 weeks	LIPUS has the potential to accelerate the osseointegration of dental implants
Zhou et al. [33]	Ovariectomized SD rats Tibiae	Length: 4.0 mm Diameter: 2.0 mm Pitch: 0.6 mm Screw-shaped	Intensity: 40 mW/cm^2 (/SATA) Pulse frequency: 1.5 MHz	20 min/day for 2, 4, 6, 8, 10, and 12 weeks	LIPUS may enhance new bone formation, especially in an early stage, and improve osseointegration in osteoporotic bone as an auxiliary method
Ruppert et al. [34]	SD rats Femur	Length: 20 mm Diameter: 1.5 mm	Intensity: 30 mW/cm^2 (/SATA) Pulse frequency: 1.5 MHz	20 min/day for 4 and 8 weeks, 5 days per week	LIPUS is superior to vibration for accelerating osseointegration and increasing bone–implant failure loads at 4 weeks
Jiang et al. [24]	αCGRP$^{+/+}$ and αCGRP$^{-/-}$ mice Maxillary first molar extraction sockets	Length: 1 mm Diameter: 0.6 mm Screw-shaped	Intensity: 30 mW/cm^2 (/SATA) Pulse frequency: 1 MHz	20 min/day for 2 and 4 weeks	LIPUS can enhance osseointegration of dental implant by inducing local neuronal production of αCGRP, providing a new idea to promote peri-implant osseointegration and bone regeneration

CGRP: calcitonin gene-related peptide; LIPUS: low-intensity pulsed ultrasound; SATA: spatial average temporal average; SD: Sprague–Dawley.

significantly than the vibration stimulator, allowing implants to achieve the stable plateau stage earlier. However, the aforementioned promoting effects disappeared after 8 weeks. In summary, the aforementioned studies showed that LIPUS strongly and stably promoted implant osseointegration and peri-implant bone regeneration, and the promoting function primarily occurred in the early period of osseointegration (about 4 weeks).

In a recent study, Jiang et al. [24] also confirmed the promoting effects of LIPUS at 30 mW/cm^2 intensity on implant osseointegration in 4 weeks, and the aforementioned function was produced through the promotion of α-calcitonin

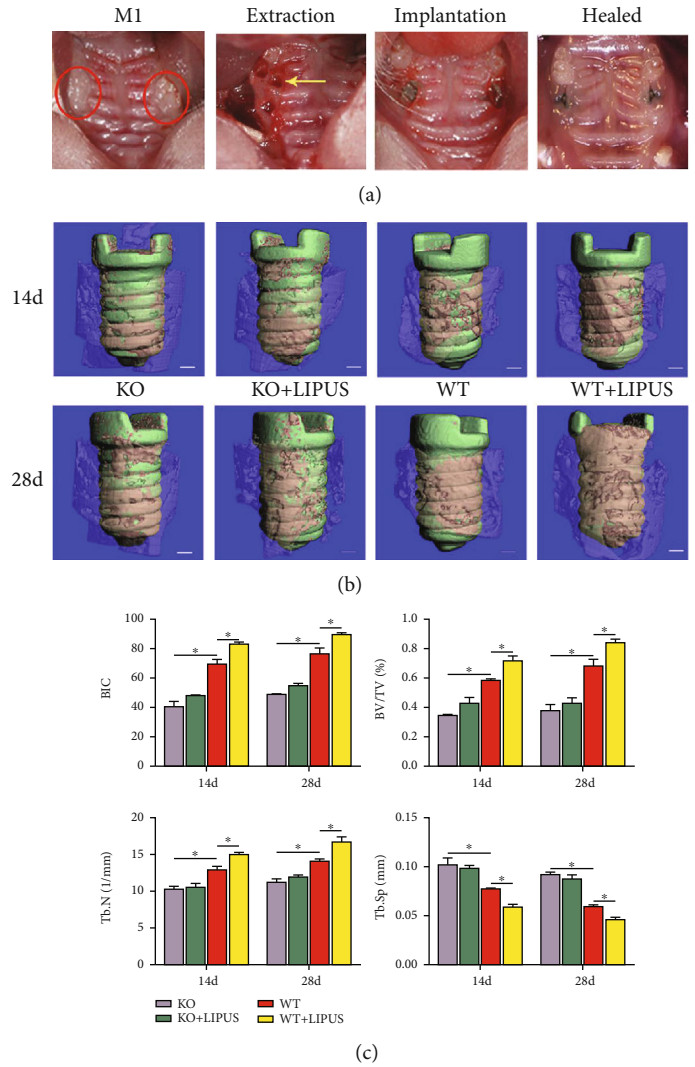

Figure 1: LIPUS enhanced the osseointegration of dental implant in αCGRP$^{+/+}$ mice. (a) Tooth extraction and implant placement procedure. The red circles indicate bilateral maxillary first molars. The yellow arrow points to the palatal root socket after tooth extraction. (b) Three-dimensional reconstruction of the implant by microcomputed tomography (micro-CT). The green area indicates implant, and the pink area indicates BIC. Scale bars = 100 mm. (c) Micro-CT analysis of BIC, BV/TV, Tb.N, and Tb.Sp. Data are presented as means ± standard deviation. $^*P < 0.05$, $n = 4$ specimens/group. KO: αCGRP knockout mice; WT: wild type; BIC: bone–implant contact; BV/TV: bone volume/tissue volume fraction; Tb.N: mean trabecular number; Tb.Sp: mean trabecular separation. Reprinted from Jiang et al. [24], Copyright (2020), with permission from Elsevier.

gene-related peptide (αCGRP) synthesis and secretion by dorsal root ganglia neurons. CGRP is a neuropeptide that regulates the biological activities of nonneural cells, and the major function of αCGRP is to regulate bone formation and remodeling [35]. Jiang et al. [24] showed that LIPUS intervention significantly promoted BIC, BV/TV, and the mean trabecular number (Tb.N) and decreased the mean trabecular separation (Tb.Sp). However, LIPUS did not have significant effects in αCGRP knockout mice (Figure 1). Therefore, αCGRP might be a hub through which LIPUS promoted implant osseointegration, and this conclusion provided a new perspective for the exploration of the mechanism of action of LIPUS.

2.2. Effect of LIPUS on Implant Osseointegration in the Presence of Osteoporosis and Diabetes Mellitus. Osteoporosis is a common human bone tissue disease primarily characterized by reduced bone density [36]. The reduction in bone mass and volume caused by bone metabolism imbalances not only affects implant osseointegration during the healing period but is also an important risk factor for a decrease in long-term implant survival rates [37, 38]. Systemic bisphosphate, estrogen, or parathyroid hormone treatment can inhibit the activity of osteoclasts in osteoporotic bone tissues and enhance osseointegration [39, 40]. However, treatment with the aforementioned systemic drugs may lead to many adverse reactions and toxic drug effects, of which osteonecrosis of the jaw bone induced by bisphosphate drugs is the most serious [41]. In this respect, LIPUS has unique advantages because it is nontoxic, nonimmunogenic, and noninvasive. Zhou et al. [33] confirmed that LIPUS could effectively promote the osseointegration of titanium

implants in osteoporotic bone tissues. Treatment with LIPUS at 40 mW/cm^2 intensity for 2 weeks significantly increased the BV/TV fraction around implants in the femurs of ovariectomized rats; treatment for 4 weeks or more significantly increased the BIC rate of the implants; and treatment for 6 weeks or more significantly increased the pullout torque. In addition, the present study confirmed that the enrichment and osteogenic differentiation of osteoblasts close to the implant–bone interface stimulated by LIPUS was an important route through which LIPUS exerted its promoting effect on osseointegration. The aforementioned results provided a theoretical basis for the application of LIPUS to assist the healing of dental implantation in patients with osteoporosis.

Diabetes mellitus is another important risk factor for implant failure during the healing period [42–44]. Many studies confirmed that the BIC rate of implants in Goto-Kakizaki (GK) rats with type 2 diabetes mellitus was significantly lower than that in normal Wistar rats, and hyperglycemia played a key role in causing the bone remodeling disorders around the implants [45–47]. In addition, cellular studies showed that a high-glucose microenvironment could significantly inhibit the proliferation and osteogenic differentiation capacity of BMSCs, reduce the expression of osteogenesis-related genes, and decelerate *in vitro* mineralization [48–50]. Unfortunately, to the best of our knowledge, currently, no relevant clinical or *in vivo* studies have reported the function and mechanism of LIPUS in the osseointegration of implants in diabetic models. However, in the field of bone fracture treatment, LIPUS significantly promoted bone healing and angiogenesis in rats with diabetes and increased the healing speed to a degree similar to that observed in normal rats [51, 52]. Based on these studies and the studies of LIPUS in the promotion of bone regeneration around implants, we speculated that LIPUS could also be used as an effective adjunct treatment method to improve implant osseointegration in patients with diabetes. However, future *in vivo* studies and clinical studies are still necessary for confirmation and more in-depth exploration.

2.3. Optimal LIPUS Treatment Parameters for Implant Osseointegration. In current studies, the parameters of LIPUS applied to dental implants, such as intensity, frequency, and intervention cycle, primarily referred to previous studies on bone fracture treatment. Since Duarte [53] used LIPUS for promoting bone fracture healing in 1983, studies on LIPUS functions mostly adopted a stimulation duration of 20 min/day. The selection of this duration not only effectively promoted bone regeneration and shortened the healing cycle but also avoided the physical and mental fatigue of patients caused by overly long intervention times. In addition, in currently published studies, the ultrasound intensity used to promote implant osseointegration and peri-implant bone regeneration was typically 30–40 mW/cm^2, and this intensity achieved good therapeutic effects [24, 27, 29, 32–34]. Nakanishi et al. [30] showed that the promoting effect of LIPUS at 40 mW/cm^2 intensity on the osseointegration and stability of implants in rabbit femurs was higher than that at 100 mW/cm^2 intensity. Similarly, cell experiments showed that LIPUS at 40 mW/cm^2 intensity induced more significant *in vitro* mineralization of the mouse osteoblast cell line MC3T3-E1 compared with 120 mW/cm^2 intensity [54]. Therefore, it was speculated that the vibration and heat generated by higher LIPUS intensities might have negative effects on the biological functions of bone-derived cells, lowering the efficacy of LIPUS compared with that at 30–40 mW/cm^2 intensity. For the ultrasound frequency, studies mainly used a fixed frequency of 1.5 MHz due to the limitation of the LIPUS instrument [27, 29, 32–34]. Only Nakanishi et al. [30] compared the effects of LIPUS on osseointegration at different frequencies, and showed that the promoting effect of LIPUS at a frequency of 3 MHz was higher than that at 1 MHz. In addition, Hsu et al. [31] applied pulsed-wave and continuous-wave ultrasound to treat implants in rabbit tibias for 30 days and showed that new bone formation around implants in the pulsed-wave groups was faster and observed more mature type I collagen expression and angiogenesis around the implants. The present study confirmed that the effect of LIPUS on implant osseointegration was better than that of low-intensity continuous ultrasound (LICUS).

The results of the aforementioned studies could be summarized as follows: LIPUS intervention at an intensity no higher than 50 mW/cm^2 (recommendation: 30–40 mW/cm^2) and a frequency of 1.5 MHz for 20 min/day for 4 weeks is currently the most commonly used and effective scheme for promoting dental implant osseointegration (Figure 2). However, this point of view still requires to be confirmed by controlled *in vivo* experiments with strict grouping in the future.

3. Effects and Associated Mechanisms of LIPUS on the Biological Functions of BMSCs

The osteogenic differentiation and bone-forming functions of bone-derived cells play key roles throughout the entire implant osseointegration process. In the early stage, after implants are installed into the jaw bone, extracellular matrix (ECM) proteins in the blood are rapidly adsorbed to the surface of implants to form a "protein layer" [55, 56]. By recognizing the Arg-Gly-Asp tripeptide sequence (RGD sequence) on the "protein layer," BMSCs and other osteogenic precursor cells begin to anchor on the surface of the implants [57], initiating subsequent proliferation and differentiation processes and synthesizing osteogenesis-associated proteins [58, 59]. Therefore, the adhesion, proliferation, and differentiation of bone-derived cells on the surface of the implant were the initial steps in the early stage of osseointegration [60].

Previous studies showed that LIPUS could be used as an effective external stimulus to improve bone regeneration around biomaterials through the promotion of proliferation and differentiation of BMSCs and osteoblasts. Moonga et al. [61] showed that LIPUS enhanced matrix mineralization of mouse MC3T3-E1 osteoblasts in bovine trabecular bone scaffold materials. Carina et al. [62] showed that LIPUS significantly promoted the osteogenic differentiation of human mesenchymal stem cells (MSCs) cultured in a mixed Mg-

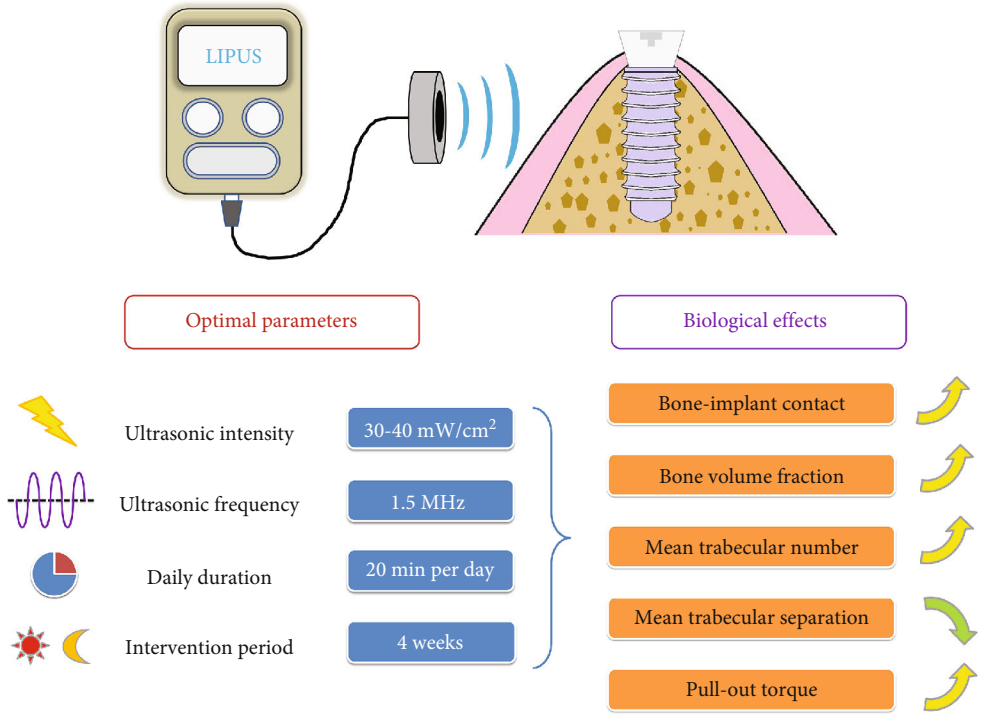

Figure 2: Schematic diagram of the optimal parameters and biological effects of LIPUS used in dental implantations. Based on the results of previous studies, LIPUS intervention at 30–40 mW/cm^2 intensity and 1.5 MHz frequency for 20 min/day for 4 weeks is the most commonly used and effective scheme for promoting dental implant osseointegration.

hydroxyapatite/collagen scaffold material. Zhou et al. [63] found that LIPUS treatment enhanced the proliferation ability of human BMSCs on 3D-bioprinted tissue scaffolds and increased the expression of alkaline phosphatase (ALP) and matrix mineralization. An et al. [64] showed that LIPUS significantly promoted the adhesion and proliferation of rat BMSCs on the surface of titanium implants, and the osteogenesis-related genes osteopontin (OPN), osteocalcin (OCN), bone morphogenetic protein-2 (BMP-2), ALP, Runt-related transcription factor 2 (Runx2), and collagen type I were upregulated under LIPUS stimulation to improve implant osseointegration.

The mechanisms through which LIPUS exerts its promoting effects on cell metabolism and tissue repair are complex and still not fully understood, but it is generally recognized that they might be associated with the mechanical stress and/or fluid microstreaming effect of LIPUS [65–68]. Ultrasonic waves can produce a weak oscillatory force resulting in potential changes in body tissues; such forces can act on the ECM, transmembrane proteins, and intracellular fluids to convert mechanical signals into biochemical signals that affect target gene expression and cellular functions [23, 69]. The already known signal transduction pathways regulated by LIPUS mainly include the integrin and focal adhesion signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, sonic hedgehog (SHH) signaling pathway, BMP/Smad signaling pathway, cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) signaling, and stromal cell–derived factor-1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR4) signaling. These signaling pathways can eventually activate bone-derived cell adhesion, migration, proliferation, and osteogenic differentiation to stimulate new bone formation and promote implant osseointegration. The mechanistic studies of these pathways can elucidate the phenomena observed during *in vivo* studies from different perspectives. The aforementioned pathways associated with the regulation of bone-derived cell biology functions and implant osseointegration promoted by LIPUS are summarized in Figure 3.

3.1. Integrin and Focal Adhesion Signaling Pathway. Integrins are a family of transmembrane proteins that mediate the connection between cells and the extracellular environment. They are also important mechanoreceptors of cells that convert the mechanical signals of LIPUS into biochemical signals. Many studies showed that LIPUS stimulation could regulate the expression level of integrin on the cell membrane. Chen et al. [70] showed that LIPUS at 60 mW/cm^2 intensity significantly increased integrin alpha 8 (ITGA8) expression in rat BMSCs and promoted the migration ability of the cells through the focal adhesion signaling pathway. Xiao et al. [71] found that after LIPUS intervention, the expression of integrin β1 in rat BMSCs increased and the cell migration ability significantly enhanced. The "ECM–integrin–focal adhesion–cytoskeleton" connection is the main pathway involved in the transmission of the LIPUS signal into cells to exert biological effects [72]. Focal adhesions are large protein complexes that connect ECM proteins and intracellular cytoskeletal proteins and are hubs for regulating cell adhesion, migration, and signal transduction [73]. When LIPUS signals are transmitted to integrin, focal adhesion kinase (FAK) is first phosphorylated to

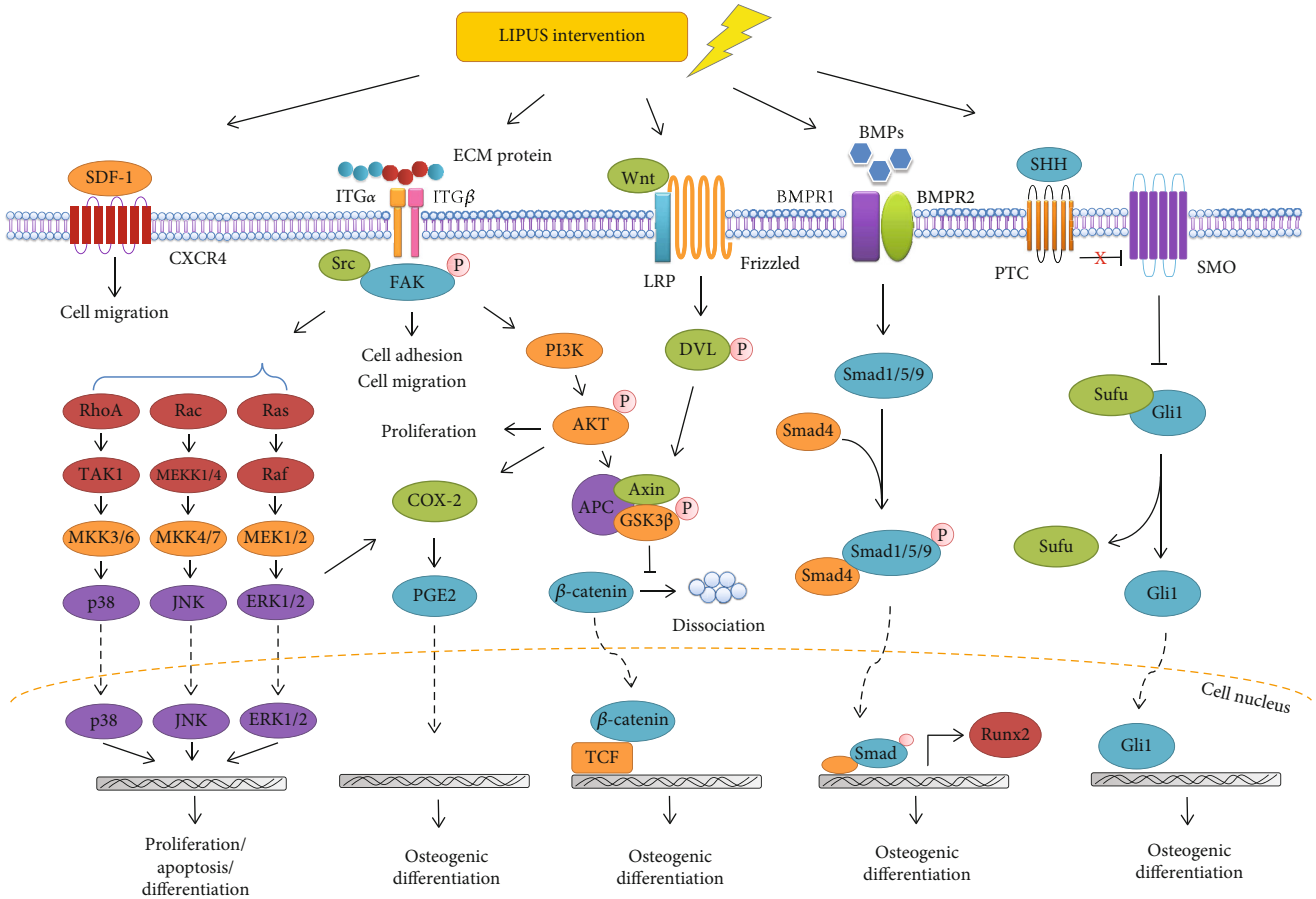

FIGURE 3: Schematic diagram of signaling pathways that can be activated by LIPUS in bone-derived cells for regulating cell biology functions and implant osseointegration, which include integrin and focal adhesion pathway, MAPK pathway, SHH pathway, BMP/Smad pathway, SDF-1/CXCR4 signaling, and COX-2/PGE2 signaling.

initiate the focal adhesion signaling pathway [74, 75], and phosphorylated FAK can activate the downstream PI3K/Akt signaling pathway to regulate the proliferation and differentiation of osteogenesis-associated cells [76].

Tang et al. [77] showed that the treatment of rat primary osteoblasts with LIPUS upregulated the expression of integrins $\alpha 2$, $\alpha 5$, $\beta 1$, and $\beta 3$ on the cell membrane and promoted osteoblast differentiation and bone formation through the ITG/FAK/PI3K/Akt signaling pathway. Xie et al. [78] showed that LIPUS treatment at 50 or 60 mW/cm^2 intensity for 5 min/day effectively promoted the proliferation ability of human BMSCs by activating the PI3K/Akt signaling pathway. Watabe et al. [79] found that LIPUS stimulation significantly upregulated integrin $\alpha 5$ (ITGA5) gene expression in mouse osteoblasts derived from the long bone, mandible, and cranial parietal bone and promoted the expression of the osteogenesis-related genes ALP and Runx2 by activating the PI3K/Akt signaling pathway.

In addition, studies found that LIPUS could also activate β-catenin signaling to significantly influence osteoblast differentiation and bone tissue regeneration [80]. Akt activation could further induce the phosphorylation of glycogen synthase kinase 3 beta (GSK3β), inactivate the APC–Axin–GSK3β complex, and inhibit the dissociation of β-catenin, causing β-catenin to accumulate and enter the nucleus to promote the transcription and synthesis of osteogenesis-associated factors [81, 82]. Thus, it was speculated that LIPUS stimulation could activate classical Wnt/β-catenin signaling through the focal adhesion signaling pathway, thus promoting new bone formation and implant osseointegration. However, the aforementioned mechanism still requires further studies for confirmation.

3.2. MAPK Signaling Pathway. MAPKs can be activated by cell stress responses induced by extracellular mechanical stimulation, mediating the transduction of mechanical signals to regulate cell proliferation and differentiation [83]. The MAPK pathway also plays an important role in the biological processes of osteoblast differentiation and bone formation [84].

The ITG/FAK/MAPK signaling pathway is a canonical pathway regulating the biological activity of bone-derived cells. FAK phosphorylation induced by LIPUS stimulation further activates three important components, extracellular signal–regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38, of the downstream MAPK pathway [85]. ERK signaling is primarily activated through the Ras/Raf/MEK/ERK pathway to regulate cell proliferation, migration,

differentiation, aging, and apoptosis [86]. Current studies generally considered that Runx2 phosphorylation could be activated by ERK signaling, which was an important mechanism underlying the promotion of osteogenic differentiation [87]. In addition, JNK activation plays a key role in cell proliferation, apoptosis, and differentiation. However, the effect of JNK on osteogenic differentiation is controversial. Some studies showed that JNK activation could inhibit the adipogenic differentiation of stem cells and promote osteogenic differentiation [88]. Other studies showed that the inhibition of JNK phosphorylation in stem cells increased the ALP expression level and promoted the osteogenic differentiation ability of the cells [89]. p38 MAPK is a stress-activated protein kinase (SAPK) that can be activated by endogenous and exogenous stimuli through MAP kinase kinase (MKK) 3/6 to participate in the stress responses of cells and regulate cell proliferation, apoptosis, and chromatin remodeling [90]. In addition, p38 activation is necessary for osteoblast differentiation [91, 92], can be activated by BMP signaling, and synergistically promotes osteoblast differentiation with Smad signaling [93, 94].

Gao et al. [95] found that LIPUS could regulate the proliferation and apoptosis of different dental stem cell populations through the MAPK signaling pathway. JNK signaling was activated by LIPUS in BMSCs, and specific inhibition of the JNK pathway blocked the promoting effect of LIPUS on cell proliferation. Kaur et al. [96] showed that LIPUS stimulated ERK1/2 activation in MC3T3-E1 mouse osteoblasts and upregulated the expression of Runx2, OCN, and OPN genes. Angle et al. [97] showed that LIPUS at 2, 15, or 30 mW/cm^2 intensities regulated the activation of ERK1/2 and p38 in rat BMSCs, thus regulating cell osteogenic differentiation. In addition, Kusuyama et al. [98] showed that LIPUS promoted the expression of Cot/Tpl2 kinase in MSCs and further regulated MEK1 and ERK phosphorylation to inhibit adipogenic differentiation and promote the osteogenic differentiation of the cells. In summary, as a group of important signaling molecules downstream of FAK, MAPK pathway members played important roles in the transition of MSCs into osteoblast cell lines under LIPUS stimulation.

3.3. SHH Signaling Pathway. The SHH signaling pathway is a classical pathway that regulates body development and homeostasis and plays an important role in bone remodeling and regeneration [99, 100]. After bone-derived cells are subjected to external stimulation, SHH in the ECM begins to interact with its membrane receptor Patched (Ptc) to relieve the inhibition on Smoothened (Smo) protein, subsequently promoting the entry of Gli protein into the nucleus to further activate the transcription of downstream osteogenesis-associated target genes [101], which directly affect the transformation of MSCs into osteoblast cell lines [102]. Zhou et al. [103] showed that LIPUS promoted the migration and proliferation of MG63 osteoblast-like cells to accelerate bone formation and the SHH inhibitor GDC0449 significantly inhibited the aforementioned functions of LIPUS. Matsumoto et al. [104] found that LIPUS significantly increased the expression of the functional genes Gli1 and Gli2 in the SHH signaling pathway and promoted the osteogenic differentiation of MC3T3-E1 cells and accelerate bone tissue regeneration by activating the SHH pathway. In addition, another study showed that activated SHH signaling could promote the osteogenesis-related gene expression of MC3T3-E1 cells by upregulating FAK phosphorylation at Tyr397 [105]. Therefore, LIPUS stimulation could not only activate the SHH signaling pathway but also interact with FAK-associated pathways to promote the osteogenic differentiation of osteoprogenitor cells and bone remodeling.

3.4. BMP/Smad Signaling Pathway. BMPs are a group of secretory proteins in the transforming growth factor-β superfamily that play critical roles in the regulation of bone metabolism [106]. After interaction with type I and type II transmembrane serine/threonine kinase receptors (BMPR-I and BMPR-II) on the cell surface, BMPs can transduce external stimulus signals into cells to regulate the osteogenic differentiation of BMSCs [107, 108]. BMP-2 is a classic osteogenesis-promoting protein. Many studies found that LIPUS could significantly promote BMP-2 synthesis and secretion in bone-derived cells to improve bone metabolism and promote bone formation [64, 109–111].

BMP-2 signaling is transmitted by intracellular signal transduction proteins called Smads. When BMP-2 interacts with its membrane receptors, Smads 1/5/9 begin to be phosphorylated and activated. Then, p-Smad 1/5/9 and Smad 4 oligomerize to form a complex and are transported into the nucleus to regulate the expression of downstream genes. Synthesis of Runx2 and many other bone formation-related factors could be stimulated by the activated BMP-2/Smad signaling pathway [106]. Maung et al. [112] showed that LIPUS significantly increased the expression of BMP-2 in periosteal cells and promoted Smad1/5/9 phosphorylation, thus enhancing the transcription of osterix (OSX) and improving the osteogenic differentiation potential of these cells. Zhang et al. [113] showed that LIPUS at 20 or 30 mW/cm^2 intensity effectively promoted BMP-2 and BMP-7 expression in stem cells, thus stimulating the osteogenic differentiation of the cells and inducing Runx2, OCN, and OPN expression by promoting Smad1/5 phosphorylation. Runx2 is a transcription factor with an important role in the bone formation process. Studies found that the expression of Runx2 in rat osteoblasts and BMSCs was significantly upregulated after LIPUS stimulation to promote the osteogenic differentiation of the cells [114–116]. Therefore, as a canonical regulatory pathway for osteogenic differentiation, the BMP/Smad/Runx2 pathway activated by the ultrasonic wave in various bone-derived cells is also an important mechanism for LIPUS to exert its biological functions.

3.5. SDF-1/CXCR4 Signaling. In the early stage of bone tissue repair or implant osseointegration, BMSCs can be recruited to the injured regions or implant sites to exert biological functions, and in this process, cell migration and chemotaxis play important roles. SDF-1 and its specific receptor, CXCR4, are key factors that regulate the migration of BMSCs to the bone remodeling site for promoting bone

fracture repair, distraction osteogenesis, extraction socket healing, and implant osseointegration [117–119]. Wang et al. [120] confirmed that LIPUS could stimulate SDF-1 secretion in stem cells and promote cell migration ability through the SDF-1/CXCR4 pathway. Xiao et al. [71] showed that LIPUS significantly promoted the migration and chemotaxis of rat BMSCs, upregulated SDF-1 and CXCR4 mRNA expression in the cells, and increased SDF-1 protein synthesis and secretion. However, after cell treatment with the SDF-1/CXCR4 pathway inhibitor AMD3100, the aforementioned functions of LIPUS were almost completely blocked. Wei et al. [121] showed that LIPUS promoted rat BMSC migration to bone tissue repair areas and observed that SDF-1 expression was upregulated in the local repair areas and the serum. Similarly, after the inhibition of SDF-1/CXCR4 signaling, the stimulatory function of LIPUS was significantly reduced. The promotion of SDF-1-mediated BMSC migration by LIPUS is also a key mechanism underlying the stimulation of implant healing because the implant osseointegration process is the process of bone tissue repair and regeneration around the implants.

3.6. COX-2/PGE2 Signaling. PGE2, a metabolite derived from arachidonic acid, has been shown to be upregulated when bone-derived cells perform their biological functions, and the expression of PGE2 is closely associated with bone remodeling and regeneration [122]. Studies showed that LIPUS could effectively increase COX-2 gene expression in osteoblasts and thereby promote PGE2 expression [123, 124]. Kokubu et al. [68] verified that COX-2 was the rate-limiting enzyme in PGE2 synthesis during LIPUS stimulation in MC3T3-E1 cells. Pretreatment of cells with specific COX-2 inhibitors could block the promoting effect of LIPUS on PGE2 expression and weaken the osteogenic ability of the cells. Naruse et al. [125] showed that the speed of bone remodeling significantly decreased in COX-2 gene knockout mice. In addition, the promotion of bone regeneration induced by LIPUS stimulation was also significantly inhibited. However, the injection of PGE2 receptor agonists restored the sensitivity of mice to LIPUS intervention. Furthermore, Hidaka et al. [126] found that LIPUS intervention increased the PGE2 level in the microenvironment of bone tissue repair areas and recruited BMSCs through PGE2 to promote local bone regeneration. Tang et al. [77] further confirmed that LIPUS stimulated COX-2 expression through the FAK/PI3K/Akt and ERK1/2 signaling pathways in MC3T3-E1 cells and upregulated PGE2 synthesis, which effectively promoted osteoblast differentiation and bone formation. Therefore, PGE2 could be directly regulated by COX-2 and might be the key target of LIPUS stimulation for bone regeneration and implant osseointegration.

Thus far, although many studies have elucidated the signaling pathways through which LIPUS regulates osteogenesis-associated cellular functions and promotes new bone formation, how these pathways interact and which pathway plays the most important role during implant healing are still not clear. In the future, *in vivo* implant models should be used for more intuitive validation and further investigation of the mechanism of action.

4. Application and Prospects of LIPUS in the Clinical Work of Dental Implantation

In recent years, LIPUS has been widely used as a convenient and effective method to promote fracture healing and bone defect repair. LIPUS was approved by the US Food and Drug Administration (FDA) as early as in 1994 and 2000 for accelerating fresh fracture healing and reconstitution of bone nonunion [127]. At present, the clinical application of LIPUS in dental implantation is still in its infancy. However, according to the existing *in vivo* studies and cell biology studies, we speculate that LIPUS may have good application value in promoting the osseointegration of implants in the future clinic practice.

In a clinical study by Abdulhameed et al. [128], LIPUS was applied to patients with dental implants in the premolar region to accelerate osseointegration. After 2 weeks of the implantation surgery, LIPUS intervention at 30 mW/cm^2 intensity and 1.5 MHz frequency was used for 10 weeks, with treatments twice a week for 20 min each time. Six months after the surgery, the clinical and imageological examinations showed that the marginal bone loss of the implants was lower in the LIPUS treatment group, vertical bone regeneration was observed, and the implant stability coefficient by resonance frequency (RF) analysis significantly increased compared with that in the conventional healing group. In another double-blind clinical study, this research group also confirmed that LIPUS stimulation could significantly improve implant stability assessed by both bone texture fractal dimension (FD) analysis and RF analysis (Figure 4) [129]. Thus, LIPUS could effectively promote implant osseointegration during the healing period and shorten the healing cycle in clinical patients. Furthermore, the aforementioned studies speculated that LIPUS could be used to save initially unstable implants and assist in obtaining higher-quality osseointegration, thus improving the success rate of implantation, especially in patients with osteoporosis and diabetes, which can affect bone remodeling.

In addition, Abdulhameed et al. observed an increase in the thickness of the buccal bone plate in the implantation area stimulated by LIPUS, with a statistically significant difference compared with the control group of patients who underwent conventional healing [128]. Moreover, Kim et al. [130] found that the local intervention with LIPUS in patients with maxillary sinus floor lift could effectively promote new bone formation, thus providing sufficient bone mass for the implant surgery in the maxillary posterior tooth area. Based on these studies, LIPUS may be used in patients undergoing bone augmentation surgery during or prior to implantation in the future to accelerate bone regeneration and shorten the treatment cycle of patients with insufficient alveolar bone for the implantation.

LIPUS has the advantages of low toxicity, low immunogenicity, noninvasiveness, high targeting selectivity, and repeatability [11, 23, 69]. The current clinical application of LIPUS has not caused any discomfort-related symptoms in patients, and no abnormal reactions, such as redness, swelling, or inflammation, have been observed in local soft tissues after the intervention. In addition, the portable

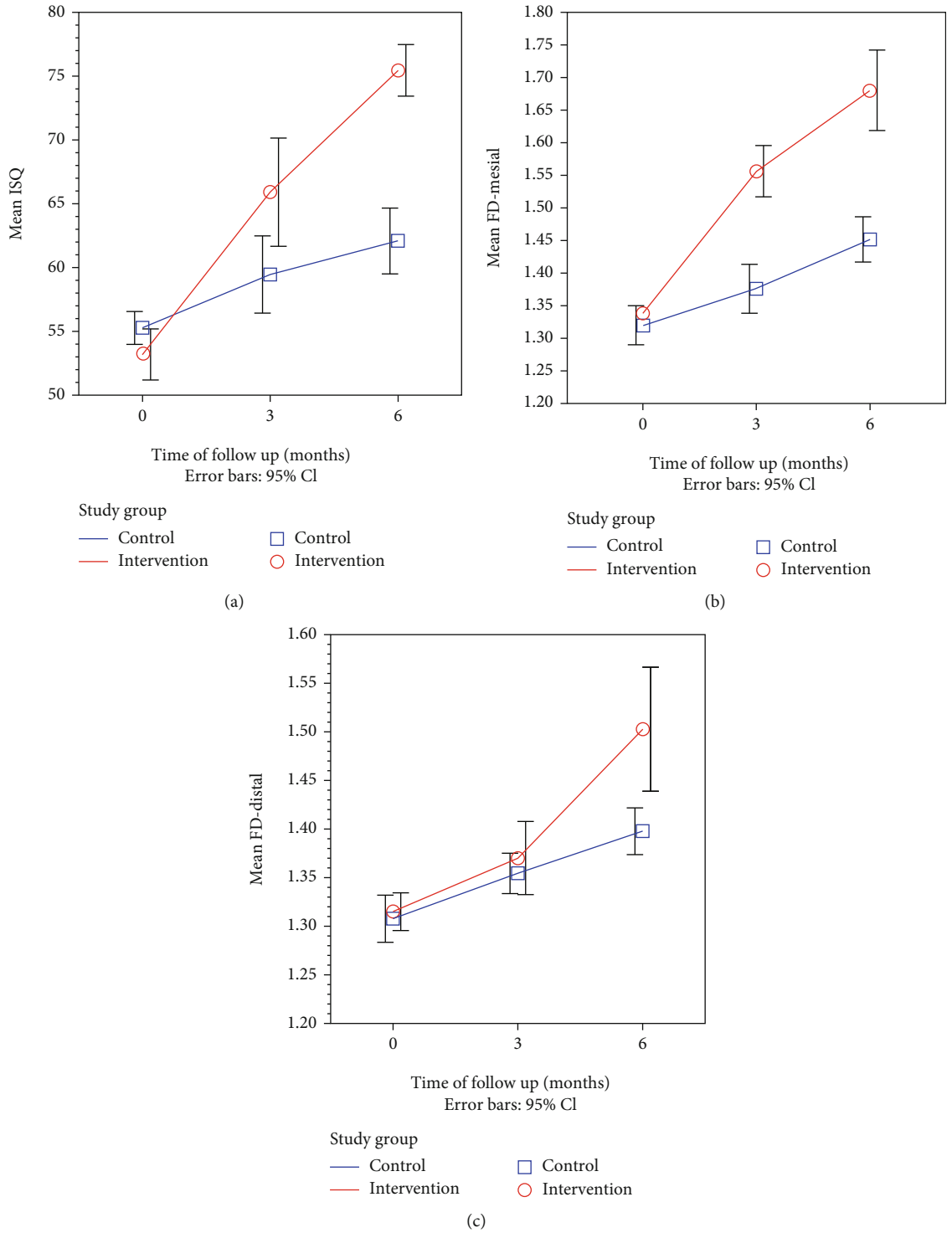

Figure 4: LIPUS stimulation could significantly improve implant stability assessed by both resonance frequency (RF) analysis and fractal dimension (FD) analysis. Line graphs showing the time trend for (a) mean RF values, (b) mesial side mean FD values, and (c) distal side mean FD values after surgery in the intervention group compared with the control group. Adapted from Abdulhameed et al. [129].

LIPUS instrument is small in size and powered by a mobile unit, and the application is not limited by space. Therefore, in the future, as an effective, safe, and comfortable physical treatment method, LIPUS may lead to the adoption of a pattern of chair-side or household treatments to assist dentists and patients in achieving higher-quality implant osseointegration, promote bone regeneration in the defect area around the implant, and even prevent marginal bone loss and improve the long-term retention rate of the implant. However, more prospective cohort studies and randomized controlled trials (RCTs) are necessary in the future to confirm the function and mechanism of LIPUS and to

determine the indications for LIPUS use in clinical practice of oral implantation, thus further supporting its application value and prospects.

5. Conclusions

As novel physiotherapy, LIPUS has been widely used in bone tissue, cartilage tissue, and soft tissue repair and reconstruction, and many studies have used it to promote the regeneration of oral and maxillofacial tissue. In the field of dental implantology, the application of LIPUS is still in its infancy. The existing studies provided a certain research foundation concerning the mechanism and clinical function of LIPUS, but further discussion is still needed.

In this review, based on the existing studies, it was found that LIPUS had an apparent promoting effect on dental implant osseointegration, suggesting that LIPUS could shorten the healing cycle after implant surgery and accelerate peri-implant bone reconstruction. Bone-derived cell adhesion, proliferation, migration, and differentiation on the surface of implants play a key role in the osseointegration process. This review systematically summarized the current role of LIPUS in the biological functions of the cells and related mechanisms. In addition, this review also addressed the application prospects of LIPUS in clinical dental implantation. Despite facing many challenges, based on the experience of LIPUS application in the treatment of bone tissue diseases, such as fracture and bone defect, the potential value of LIPUS in clinical dental implantation may be far beyond the existing reports.

Based on the in-depth exploration of the mechanism of LIPUS *in vitro*, we suggested that a transformation from a small-animal model to a large-animal model be considered for *in vivo* validation experiments. A model of jaw bone implantation with weight-bearing stress can be established, and the observation period can be further extended to more convincingly detect the effect of LIPUS. In addition, we also suggest exploring the therapeutic effect of LIPUS on the osseointegration of implants in abnormal microenvironments, such as in diabetes or osteoporosis, and clarifying the intervention effect of LIPUS on implants with poor initial stability or a poor healing state, so as to provide a new theoretical basis for improving the success rate of dental implantation and expanding the clinical indications.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Endothelial Cells Promote Migration of Mesenchymal Stem Cells via PDGF-BB/PDGFRβ-Src-Akt in the Context of Inflammatory Microenvironment upon Bone Defect

Sihao He,[1,2,3] **Tianyong Hou**,[1,2,3] **Jiangling Zhou**,[1,2,3] **Qiuchi Ai**,[1,2,3] **Ce Dou**,[1,2,3] **Fei Luo**,[1,2,3] **Jianzhong Xu**,[1,2,3] **and Junchao Xing**[1,2,3]

[1]*National Regional United Engineering Laboratory of Tissue Engineering, Department of Orthopedics, Southwest Hospital, Army Military Medical University, Chongqing, China*
[2]*Center of Regenerative and Reconstructive Engineering Technology in Chongqing City, Chongqing, China*
[3]*Tissue Engineering Laboratory of Chongqing City, Chongqing, China*

Correspondence should be addressed to Jianzhong Xu; prof_jianzhongxu@163.com and Junchao Xing; 46327289@qq.com

Sihao He and Tianyong Hou contributed equally to this work.

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Homing of mesenchymal stem cells (MSCs) to the defect site is indispensable for bone repair. Local endothelial cells (ECs) can recruit MSCs; however, the mechanism remains unclear, especially in the context of the inflammatory microenvironment. This study was aimed to investigate the role of ECs in MSCs migration during the inflammatory phase of bone repair. The inflammatory microenvironment was mimicked *in vitro* via adding a cytokine set (IL-1β, IL-6, and TNF-α) to the culture medium of ECs. The production of PDGF-BB from ECs was measured by ELISA. Transwell and wound healing assays were employed to assess MSCs migration toward ECs and evaluate the implication of PDGF-BB/PDGFRβ. A series of shRNA and pathway inhibitors were used to screen signal molecules downstream of PDGF-BB/PDGFRβ. Then, mouse models of femoral defects were fabricated and DBM scaffolds were implanted. GFP$^+$ MSCs were injected via tail vein, and the relevance of PDGF-BB/PDGFRβ, as well as screened signal molecules, in cell homing was further verified during the early phase of bone repair. In the mimicked inflammatory microenvironment, MSCs migration toward ECs was significantly promoted, which could be abrogated by *pdgfrb* knockout in MSCs. Inhibition of Src or Akt led to negative effects analogous to *pdgfrb* knockout. Blockade of JNK, MEK, and p38 MAPK had no impact. Meanwhile, the secretion of PDGF-BB from ECs was evidently motivated by the inflammatory microenvironment. Adding recombinant PDGF-BB protein to the culture medium of ECs phenocopied the inflammatory microenvironment with regard to attracting MSCs, which was abolished by *pdgfb*, *src*, or *akt* in MSCs. Moreover, *pdgfb* knockout suppressed the expression and phosphorylation of Src and Akt in migrating MSCs. *Src* knockout impaired Akt expression but not vice versa. *In vivo*, reduced infiltration of CD31$^+$ ECs was correlated with diminished PDGF-BB in local defect sites, and silencing *pdgfb*, *src*, or *akt* in MSCs markedly hampered cell homing. Together, these findings suggest that in the inflammatory microenvironment, MSCs migrate toward ECs via PDGF-BB/PDGFRβ and the downstream Src-Akt signal pathway.

1. Introduction

Large segmental bone defects caused by trauma, tumor resection, or bone infection remain among the most prevalent clinical challenges. The *in situ* tissue engineering concept, which is based on attracting and modulating osteoprogenitors already present in a patient's body to accelerate bone repair, has become a promising strategy. The reparative specialty of resident mesenchymal stem cells (MSCs) is worthless unless their directional homing is

appropriately controlled [1]. Physiologically, MSCs reside in the bone marrow niche, and their engagement and disengagement maintain a dynamic balance. Upon injury, an inflammatory microenvironment is triggered locally and the balance is disrupted, leading to abundant cell egression into circulation and migration to the injury sites [2]. Meanwhile, local vascularization is reinforced in response to inflammation. During bone repair, angiogenesis, the formation of new blood vessels from preexisting ones, is closely coupled with osteogenesis [3]. To a certain extent, the onset time and extent of revascularization determine the outcome of bone grafting [4]. Therefore, the inflammatory microenvironment and vascularization situation are critical factors for effective MSCs homing. Nevertheless, the precise functional modes and mechanisms remain confused.

During the embryonic and postnatal periods, intimate physical proximity exists between blood vessels and osteoprogenitors, implying a close relationship between endothelial cells (ECs) and MSCs [5]. Indeed, the crosstalk of ECs and MSCs has been widely documented and exploited to ameliorate blood supply and expedite tissue regeneration [6]. Initially, attention was given to the transdifferentiation of MSCs into ECs or the regulatory effects of MSCs on ECs [7]. For example, we and others have reported that MSCs can attract endothelial lineages via the chemokine-receptor cascade reactions [8, 9]. Currently, there is growing interest in the inversus effects. Recent findings suggest that a certain subset of ECs, mainly referred to as Type-H ECs ($CD31^{hi}Emcn^{hi}$), precedes and guides homing of osteoprogenitors [3]. Reduction of EC infiltration by VEGFR2 antagonist impedes migration of osteoprogenitors and bone reconstruction. ECs can facilitate tissue regeneration through their paracrine capacity [10], by which they communicate with osteoprogenitors. The secretome of ECs is influenced by the local microenvironment, including inflammation. Among EC-derived biologics, platelet-derived growth factor (PDGF) family is famous for regulating the viability and proliferation of MSCs [11]. Thereinto, the PDGF-BB homodimer appears to be highly potent in fostering osteogenesis via activation of multiple kinase-dependent signaling cascades [12]. Besides, the ability of PDGF-BB in promoting cell migration has been widely documented [13]. The major receptor, platelet-derived growth factor receptor-β (PDGFRβ), is believed to mobilize cells of mesenchymal origin [13].

Here, we tried to investigate the chemotactic effects of ECs on MSCs in the early inflammatory microenvironment following bone defects, as well as the involvement of PDGF-BB/PDGFRβ, aiming to shed light on the early angio-osteogenic coupling and provide therapeutic targets for *in situ* bone tissue engineering. First, the inflammatory microenvironment was mimicked *in vitro* as reported previously [14]. Next, MSCs movement toward ECs was evaluated via migration assays. The relevance of PDGF-BB/PDGFRβ was defined via gene silence and pathway inhibitors. Then, signal molecules downstream of PDGF-BB/PDGFRβ were screened out *in vitro* and further validated *in vivo*. Eventually, we concluded that in the inflammatory microenvironment, ECs promoted MSCs migration via the PDGF-BB/PDGFRβ-Src-Akt pathway.

2. Materials and Methods

2.1. Cell Culture. All experiments on human and animal samples were approved by the Ethics Committee, Southwest Hospital, Army Military Medical University. Human bone marrow MSCs (hBMSCs) and human umbilical vein endothelial cells (HUVECs) were purchased from Cyagen Biosciences (HUXMA-01001, HUVEC-20001). hBMSCs were cultured in basic culture medium containing Dulbecco's modified Eagle's medium/F12 (DMEM/F12; 1 : 1; HyClone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 100 U/ml penicillin/streptomycin (Gibco, USA). HUVECs were cultured in Endothelial Cell Growth Medium-2 (EGM; CC-3162; Lonza, Switzerland) containing 10% FBS and 100 U/ml penicillin/streptomycin. The media were changed every other day. When reaching 80-90% confluence, cells were digested using 0.25% trypsin-EDTA (Gibco, USA) and passaged. HUVECs at passage 3 and hBMSCs at passage 4 were harvested for use.

Mouse mesenchymal stem cells (mBMSCs) were isolated and cultured as described previously [9]. Briefly, bone marrows were extracted from femurs by resecting the epiphyses and flushing the shaft with cold phosphate buffered saline (PBS; Beyotime, China). Cells were collected by centrifugation and resuspended in basic culture medium containing DMEM/F12 supplemented with 15% FBS and 100 U/ml penicillin/streptomycin. Then, cells were incubated in a 5%-CO_2 incubator at 37°C. After 24 h, nonadherent cells were discarded and the culture media were changed every 48-72 h. When reaching confluence of more than 80%, cells were trypsinized and passaged for 3 times before use.

2.2. Preparation of Conditioned Media. HUVECs were incubated with EGM added with or without 4 ng/ml IL-1β, 10 ng/ml IL-6, and 20 ng/ml TNF-a (all from PeproTech, Rocky Hill, NJ, USA) to prepare conditioned media of inflammatory ECs (IEC-CM) or ECs (EC-CM). After 48 h, the supernatants were collected, centrifuged, aliquoted, and stored at -80°C. EGM free of serum and supplemented with stromal cell-derived factor 1 (SDF-1; 100 ng/ml) served as negative and positive controls, respectively. The contents of PDGF-BB in IEC-CM and EC-CM were measured using a human ELISA kit (Solarbio, Beijing, China) according to the manufacturer's instructions.

2.3. Gene Interference. HUVECs, hBMSCs, and GFP^{+-} mBMSCs (obtained from GFP transgenic C57 mice) were infected with lentivirus particles encoding the corresponding short hairpin RNA (shRNA; Santa Cruz Biotechnology, Dallas, TX, USA) according to the manufacturer's instructions. Clones expressing the virus were selected by their resistance to puromycin (Sigma-Aldrich, St. Louis, Missouri, USA). The interference efficiency was confirmed by western blot.

2.4. Migration Assay. Transwell inserts with 8-mm pores (Corning, NY, USA) were used for *in vitro* migration assays. Conditioned medium (700 ml) was added to the bottom compartment. hBMSCs were pre-treated with serum-free medium or medium supplemented with inhibitors, as detailed in Table 1. The upper chamber of Transwell insert

Table 1: Reagent information.

Reagent	Target	Concentration	Duration	Usage	Source
AMD3100	CXCR4	5 μg/ml	30 min	Pre-treat MSCs	Sigma-Aldrich, St. Louis, MO, USA
AZD0530	Src	10 μM	24 h	Pre-treat MSCs	Selleck Chemicals, Houston, TX, USA
MK-2206	Akt1/2/3	10 μM	24 h	Pre-treat MSCs	Selleck Chemicals, Houston, TX, USA
U0126	MEK1/2	50 μM	24 h	Pre-treat MSCs	Selleck Chemicals, Houston, TX, USA
SP600125	JNK1/2/3	10 μM	24 h	Pre-treat MSCs	Selleck Chemicals, Houston, TX, USA
SB203580	p38 MAPK	1 μM	30 min	Pre-treat MSCs	Selleck Chemicals, Houston, TX, USA
JNJ-10198409	PDGFRβ	5 μM	1 h	Pre-treat MSCs	MedChemExpress, Monmouth Junction, NJ, USA
SU5408	VEGFR2	5 μM	1/2d since implantation	Intraperitoneal injection	MedChemExpress, Monmouth Junction, NJ, USA

was filled with 5×10^4 cells, which were allowed to migrate at 37°C. Cells in partial groups were collected for biochemical analysis at 30 min. After 8 h, hBMSCs on the top side of the insert (non-migrating cells) were dislodged with a cotton tip applicator. Then, the migrated cells on the bottom side were washed with PBS, followed by fixation with 4% paraformaldehyde (Boster Biologic Technology, Wuhan, China). The membrane was moved onto one object slide with the lower side upward. Cells retained on the membrane were labeled with DAPI (Invitrogen, Carlsbad, CA, USA) and subjected to fluorescent microscopy. For every group, three high-power fields (HPF, ×200) were randomly chosen and migrated hBMSCs were counted and averaged. Migration assay was repeated in triplicate.

For the wound healing assay, groups of hBMSCs were seeded and cultured in 6-well plates (1×10^5/well) to reach the confluent monolayer. Then, cells were scraped using a 200 μL pipette tip and washed with PBS to clear cell debris and suspension. Complete medium was replaced with conditioned media and cells were incubated for 12 h. Microscopic images were captured at the same position of the wound at 0 and 12 h. Migration ability was measured by the rates of scratch wound closure using the ImageJ software (National Institutes of Health, Maryland, USA).

2.5. RT-PCR. Primers are shown in Table 2. Total RNA was extracted using TRIzol reagents (Invitrogen, Carlsbad, CA). cDNA was prepared using a cDNA Synthesis Kit (TaKaRa, Japan) and RT-PCR was implemented with a QuantiTect™-SYBR Green PCR Kit (TaKaRa, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a reference control. Reactions were repeated in triplicate.

2.6. Western Blot. SDS lysis buffer (100 mM Tris at pH 8.0, 10% glycerol, and 1% SDS) was used for cell lysis. Using a NanoVue spectrophotometer (GE Healthcare, Waukesha, WI, USA), the protein concentration was measured. For each sample, protein lysate (30 mg) was isolated by SDS-PAGE (120 min, 80 V; Beyotime, Shanghai, China) and electrotransferred to the polyvinylidene fluoride membrane (60 min, 250 mA; Millipore, MA, USA). After blocking using milk (5%), each membrane was incubated at 4°C for 12 h with the corresponding primary antibodies, which are detailed in Table 3. Following a thorough wash with TBST, the blots were incubated with the secondary antibody (horseradish peroxidase-conjugated, 1 : 2000; Southern Biotech, AL, USA) at room temperature (RT) for about 1 h. The membranes were visualized by ECL (Kirkegaard&Perry Lab, MD, USA). GAPDH was used as control. All experiments were repeated for 3 times.

2.7. Animal Manipulation. Decalcified bone matrices (DBM) were prepared from the trabecular bones of Yunnan miniature pigs and surgeries were performed according to procedures reported previously [15]. Femoral critical-sized bone defects (2 mm in length) were created in C57 mice (8weeks, male) and DBM were implanted. Implants harvested at postoperative days 1, 3, and 7 were subjected to fluorescence-activated cell sorting (FACs), RT-PCR, and western blot. At 7 days, wild GFP^+ mBMSCs or cells intervened by shRNA were injected (1×10^6/mouse) via tail vein every 2 days (Supplemental Figure 1) [16, 17].

2.8. FACs. Cells were harvested from implants by sufficient digestion, which was achieved by using Type I collagenase (1 mg/ml) and trypsin (0.25%) plus EDTA (0.01%; Thermo Fisher Scientific, MA, USA). Then, cells in each group were filtered and centrifuged. After resuspension in PBS containing 2% FBS, cells were incubated with the antibody against CD31 (fluorescence-conjugated; BD Biosciences, CA, USA) at 4°C for 30 min. Non-stained cells were incubated with the isotype control. After centrifugation and resuspension in propidium iodide, the sample was subjected to a CytoFLEX S Flow Cytometer (Beckman Coulter, CA, USA). Data were inspected with the CytoFlex software. For each group, the experiment was repeated in triplicate.

2.9. Immunofluorescent Staining. Three mice from each group were euthanized. The femurs were collected, fixed with paraformaldehyde (4%) for 24 h, and decalcificated using EDTA for 7-10 days. Then, 7-mm-thick frozen sections were prepared and permeabilized using Triton X-100 (0.3%), followed by blocking with donkey serum (1 : 20; Huayueyang Biotech, Beijing, China). Subject slides were

Table 2: Primers for RT-PCR.

Gene	Species	Forward (5′-3′)	Reverse (5′-3′)
Src	Human	AAGCTGAGGCATGAGAAG	GTACTCCGTGACGATGTAA
Akt	Human	TATTGTGAAGGAGGGTTG	ATTCTTGAGGAGGAAGTAG
GAPDH	Human	ATCAACTCACCGCCAACA	CGACTCAATCTTCCTCTCCAG
PDGF-BB	Mouse	CATCGAGCCAAG ACACCTCA	AGTGCCTTCTTGTCA TGGGT
GAPDH	Mouse	CGGATTTGGTCGTATTGG	TCCTGGAAGATGGTGATG

Table 3: Antibody information.

Antibody	Usage	Host-reactivity	Dilutions	Clonality	Source
PDGF-BB	Western blot	Rabbit anti-mouse	1 : 500	Polyclonal	Abcam, Cambridge, UK
PDGF-BB	Western blot	Chicken anti-human	1 : 500	Polyclonal	Abcam, Cambridge, UK
PDGFRβ	Western blot	Rabbit anti-mouse/ human	1 : 1000	Monoclonal	Cell Signaling Technology, Danvers, MA, USA
Src	Western blot	Rabbit anti-mouse/ human	1 : 1000	Monoclonal	Cell Signaling Technology, Danvers, MA, USA
p-Src	Western blot	Rabbit anti-mouse/ human	1 : 1000	Monoclonal	Cell Signaling Technology, Danvers, MA, USA
Akt	Western blot	Rabbit anti-mouse/ human	1 : 1000	Monoclonal	Cell Signaling Technology, Danvers, MA, USA
p-Akt	Western blot	Rabbit anti-mouse/ human	1 : 2000	Monoclonal	Cell Signaling Technology, Danvers, MA, USA
CD31	Immunofluorescence	Goat anti-mouse	1 : 100	Polyclonal	R&D Systems, Minneapolis, MN, USA
PDGFRβ	Immunofluorescence	Goat anti-mouse	1 : 100	Polyclonal	R&D Systems, Minneapolis, MN, USA
Anti-GFP	Immunofluorescence	Rabbit anti-GFP	1 : 200	Polyclonal	Abcam, Cambridge, UK
Alexa Fluor® 488	Immunofluorescence	Goat anti-rabbit	1 : 200	Polyclonal	Abcam, Cambridge, UK
NL557	Immunofluorescence	Donkey anti-goat	1 : 500	Polyclonal	R&D Systems, Minneapolis, MN, USA

incubated with primary antibodies (Table 3) at 4°C for 12 h. Then, samples were stained with the corresponding secondary antibodies (Table 3) for 1 h and counterstained with DAPI for about 10 min. Randomly, three separate sections were selected from more than 20 sections for each group. Relative cellularity was measured by counting stained cells in five HPF using a confocal laser scan microscope (Leica Biosystems, Wetzlar, Germany).

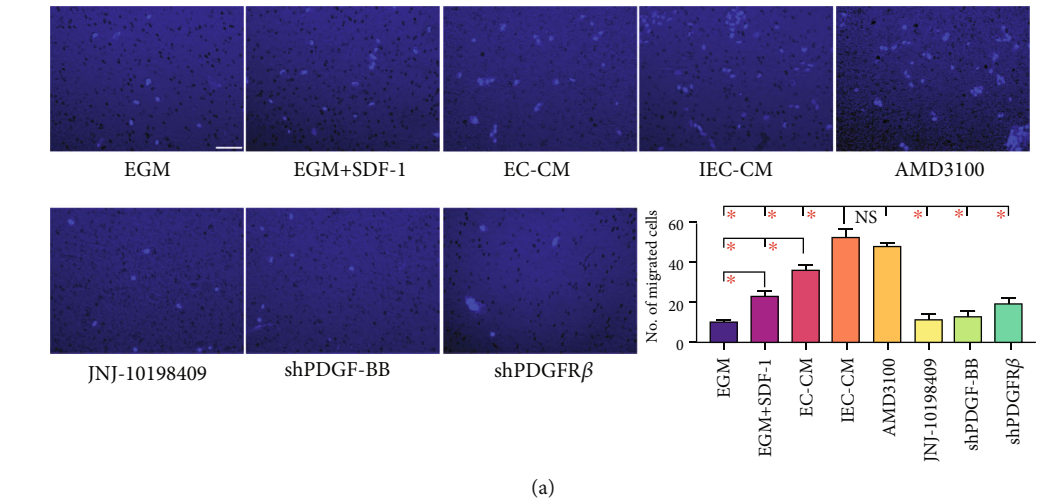
2.10. Histological Observation. At 4 weeks postoperatively, the implants were obstained, decalcified with EDTA (10%), dehydrated in graded alcoholic solutions, and embedded in paraffin. 7-mm-thick sections were prepared and stained using by hematoxylin and eosin (HE) and Masson's trichrome methods. Using a microscope (Olympus, Hamburg, German), images were taken.

2.11. Statistical Analysis. Data were presented as means ±SEM. For ELISA, RT-PCR, western blot, FACs, and migration assay, one-way ANOVA followed by SNK test was conducted to determine the statistical significance between groups (SPSS v.13.0). The correlation analysis was identified by Pearson's correlation coefficient. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. ECs Promote MSCs Migration in the Inflammatory Microenvironment. Compared with control, EC-CM and IEC-CM significantly promoted MSCs migration, and IEC-CM showed the strongest chemotactic power (Figure 1(a)). SDF-1 and its receptor CXCR4 have been widely documented to be indispensable for MSCs migration. Yet, the chemotactic power of EC-CM and IEC-CM was much greater than that of SDF-1(Figure 1(a)). Meanwhile, AMD3100, a specific CXCR4 antagonist, had no effect on IEC-CM-induced migration. Consistent findings were obtained from the wound healing assay (Figure 1(b)). Although both EC-CM and SDF-1 induced MSCs migration, IEC-CM showed the highest rate of scratch area closure. Together, these findings reinforced the recruiting effect of ECs on MSCs, which could be remarkably enhanced by the inflammatory microenvironment.

3.2. MSCs Migrate toward ECs via PDGF-BB/PDGFRβ in the Inflammatory Microenvironment. Other than SDF-1/CXCR4, signaling pathways mediated by PDGF-BB/PDGFRβ possess a crucial role in stem cell motility. To unveil the mechanism underlying MSCs migration toward ECs in the inflammatory microenvironment, PDGFRβ was



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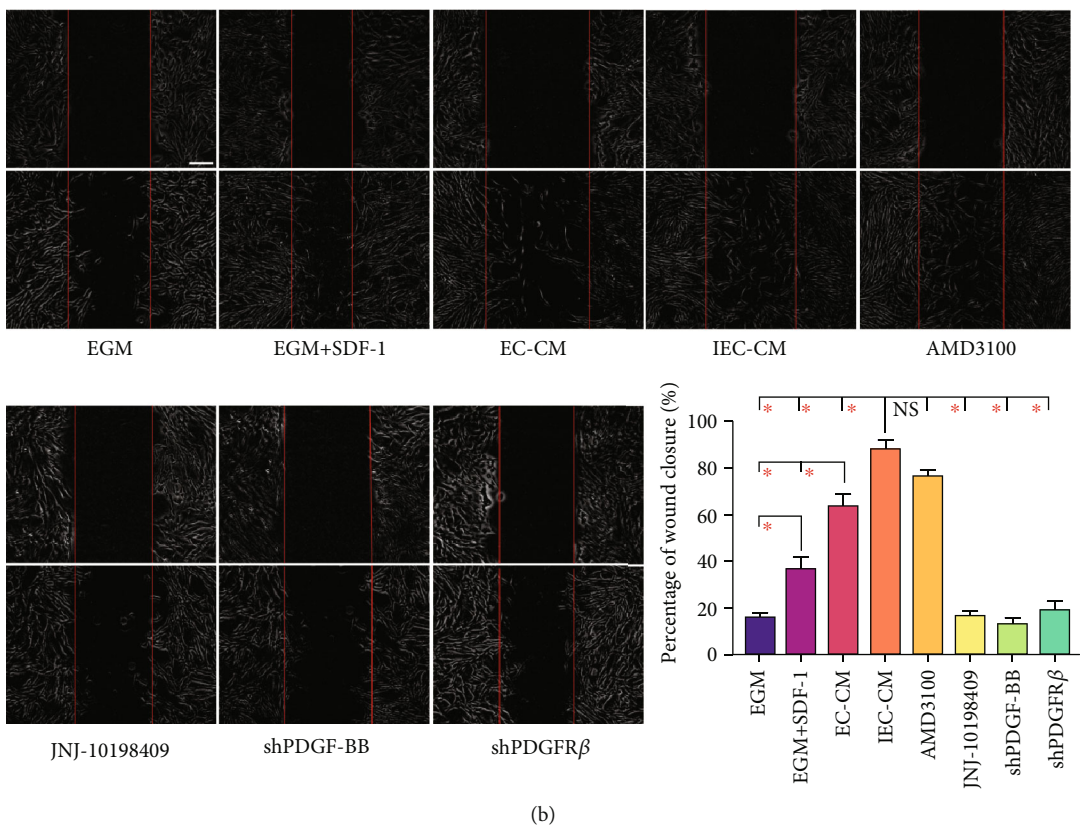


(b)

Figure 1: MSCs migrated toward ECs via PDGF-BB/PDGFRβ in the inflammatory microenvironment. (a) Representative images of migrated hBMSCs that had received different pre-treatments or had been exposed to different inducing media. The migration capacity of hBMSCs was determined using a Transwell culture system. The quantification of migrated cells was shown as a bar graph. Scale bar, 50 μm. $*P < 0.05$. (b) Representative images of wound healing assays. The rate of scratch wound closure was shown as a bar graph. Scale bar, 200 μm. $*P < 0.05$. EGM: endothelial cell growth medium-2. EC-CM: conditioned media of endothelial cells. IEC-CM: conditioned media of endothelial cells in the context of inflammatory microenvironment; shPDGF-BB: short hairpin RNA targeting *pdgfb*; shPDGFRβ: shRNA targeting *pdgfrb*.

firstly blocked in MSCs using a specific inhibitor, JNJ-10198409. MSCs migration toward IEC-CM was abrogated by JNJ-10198409 (Figure 2(a)), underlining the relevance of PDGFRβ. Then, the concentration of PDGF-BB in the conditioned media was measured. ECs secreted PDGF-BB spontaneously, and the inflammatory microenvironment memorably forced the production (Supplemental Figure 2A). Thereafter, genes of *pdgfb* and *pdgfrb* were knocked out by shRNA in cultured ECs and MSCs, respectively. The interference efficiencies of shRNA were checked by western blot. Sh-1 for PDGF-BB and sh-2 for PDGFRβ were chosen for use (Supplemental Figure 2B, 2C). As a result, MSCs migration toward IEC-CM was dramatically impeded by shRNA targeting PDGF-BB in

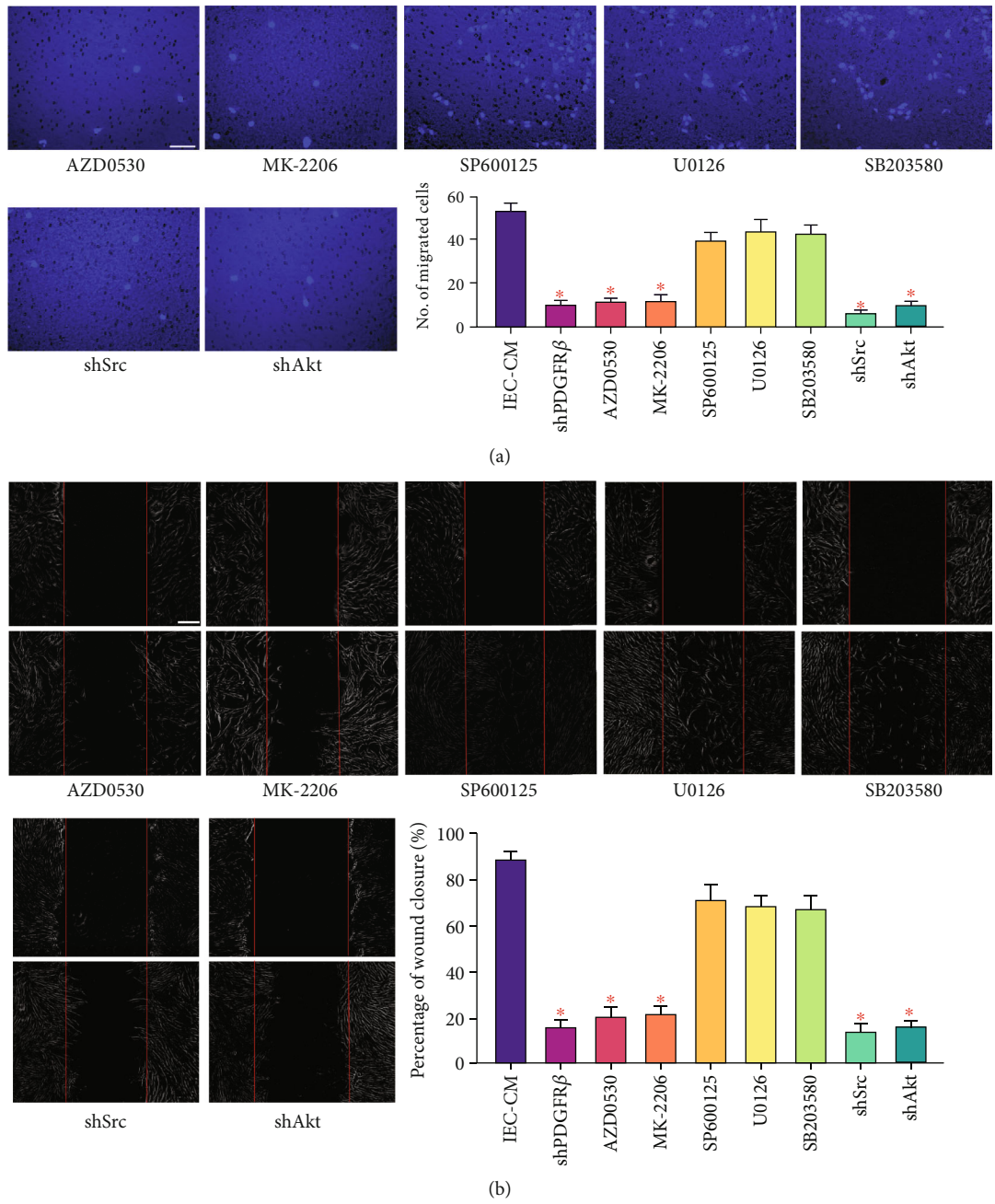


Figure 2: Src and Akt were required for ECs-induced MSCs migration in inflammatory microenvironment. (a) Representative images of migrated hBMSCs in Transwell culture systems. Pathway inhibitors were used for pre-treating cells migrating to IEC-CM. The quantification of migrated cells was shown as a bar graph. Data were compared with the groups of IEC-CM and shPDGFRβ from Figure 1. Scale bar, 50 μm. $*P < 0.05$. (b) Representative images of wound healing assays. The rate of scratch wound closure was shown as a bar graph. Data were compared with the groups of IEC-CM and shPDGFRβ from Figure 1. Scale bar, 200 μm. $*P < 0.05$. IEC-CM: conditioned media of endothelial cells in the context of inflammatory microenvironment; shPDGFRβ: short hairpin RNA targeting *pdgfrb*; shSrc: shRNA targeting *src*; shAkt: shRNA targeting *akt*.

ECs or PDGFRβ in MSCs (Figure 1(a)). Furthermore, the wound healing assay verified the reduced ability of MSCs in repairing the damaged area after gene silence (Figure 1(b)). These results collectively suggested that ECs-induced MSCs migration in the inflammatory microenvironment was attributed to the activation of PDGF-BB/PDGFRβ.

3.3. Src and Akt Function Downstream of PDGFRβ during MSCs Migration toward ECs. In terms of cell migration, various signaling molecules have been identified to be associated with PDGF-BB/PDGFRβ, such as phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt), c-JunN-terminal kinase (JNK), mitogen-activated protein (MEK), mitogen-activated protein kinase (MAPK), and steroid receptor

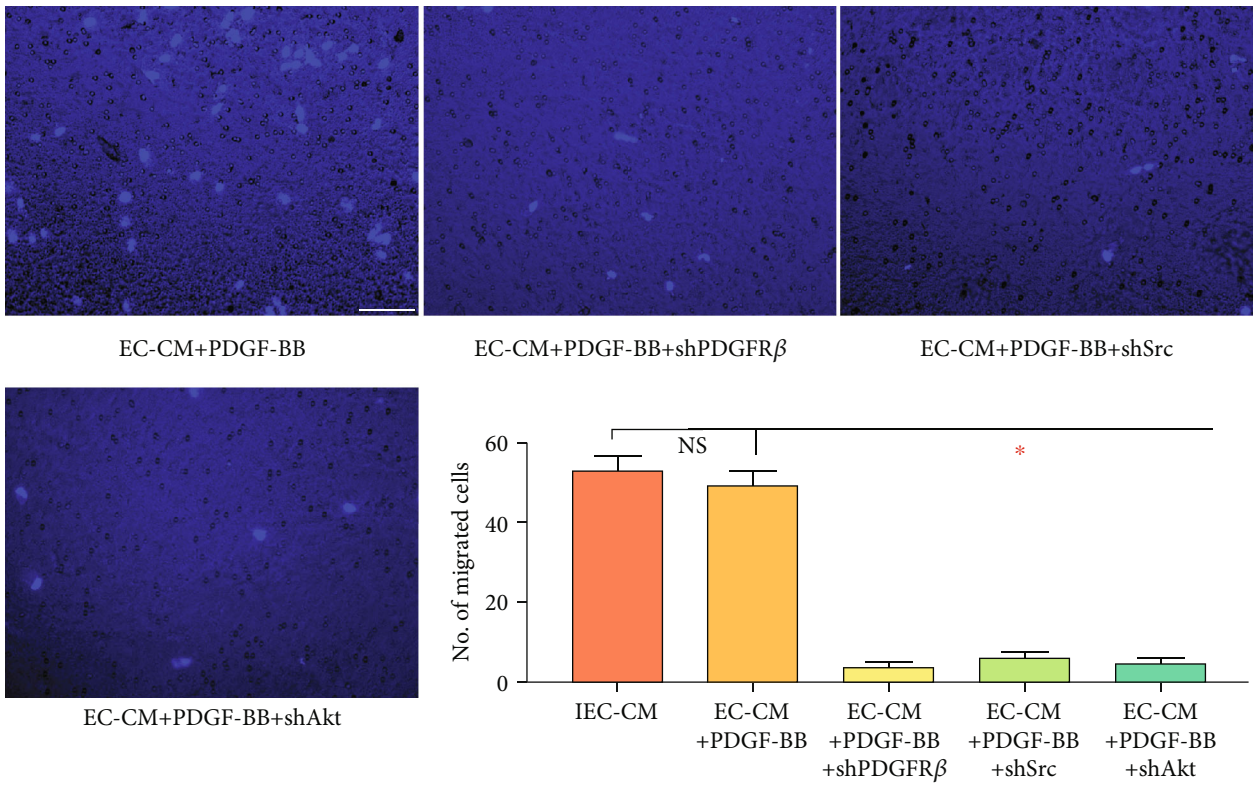
coactivator (Src). Next, we tried to screen signals downstream of PDGFRβ, based on a series of highly selective pathway inhibitors. Accordingly, pre-treating MSCs with an inhibitor of JNK (SP600125), MEK (U0126), or p38 MAPK (SB203580) only slightly weakened migration toward IEC-CM (Figure 2(a)). In contrast, AZD0530 (Src) or MK-2206 (Akt) led to a remarkable migratory energy in a manner similar to *pdgfrb* interference, indicating the implication of Src and Akt. Then, genes of *src* and *akt* were knocked out in MSCs (Supplemental Figure 2C) and markedly suppressed cell migration toward IEC-CM (Figure 2(a)). Analogical findings were obtained from the wound healing assay (Figure 2(b)). MSC movement to the scratch region, induced by IEC-CM, was significantly inhibited after knockout of *pdgfrb*, *src*, or *akt*. Nevertheless, blockade of JNK, MEK, or p38 MAPK showed no obvious difference. To figure out the relationship of Src and Akt with PDGF-BB, recombinant PDGF-BB protein was added to EC-CM. PDGF-BB elevated the chemotctic power of EC-CM to a level similar to that of IEC-CM (Figure 3). The augmentative effect of PDGF-BB was abrogated by knockout of *pdgfrb*, *src*, or *akt* in MSCs. Collectively, these findings suggested that Src and Akt were effectors downstream of PDGF-BB/PDGFRβ.

For further verification, *in vivo* experiments were performed. $CD31^+$ ECs that infiltrated in the implantation area were sorted and the expression of PDGF-BB was detected. The ratio of ECs gradually increased over time (Figure 4(a)). Similar variation trend was gained in the mRNA and protein expression of PDGF-BB (Figure 4(b)). Moreover, a positive correlation existed between the ratio of $CD31^+$ ECs and the protein level of PDGF-BB (Pearson's correlation coefficient $R=0.926$, $P<0.05$; Figure 4(c)). At 7 days, administration of the VEGFR2 specific inhibitor, SU5408, significantly impeded the infiltration of $CD31^+$ ECs within implants (Figure 4(d)). In consequence, the concentration of PDGF-BB within implants was sharply reduced to an extremely low level. According to the current literature, VEGF-mediated activation of VEGFR2 suppressed PDGFRβ signaling in vascular smooth muscle cells through the assembly of the receptor complex consisting of VEGFR2 and PDGFRβ [18]. Limited by the accessible evidence, the potential effect of SU5408 on the expression of PDGF-BB could not be entirely excluded. Considering that ECs are one of the main sources of PDGF-BB, these findings indirectly suggested that at least in the early inflammatory phase (<7 days), the infiltrated ECs within implants served as an important source of PDGF-BB at local sites. Then, GFP^+ mBMSCs with gene interference (*pdgfb*, *src*, or *akt*, Supplemental Figure 2D) were administrated via tail intravenous injection. At 10 days, GFP^+ cells appeared in the graft area and almost all of them expressed PDGFRβ in the control group (Figure 4(e)). In contrast, knockout of *pdgfb*, *src*, or *akt* resulted in a dramatic decrease in the number of GFP^+ cells within implants. It was notable that although the amount was small, GFP^+ cells were present after *pdgfb* knockout but they seldom expressed PDGFRβ. This might be ascribed to the fact that the interference efficiency of PDGFRβ was not 100% and the participation of other pathways guiding MSCs homing. Intriguingly, GFP^+ cells were almost invisible after *src* knockout, but $PDGFR\beta^+$ cells were evident, indicating the predominant role of Src in MSCs homing. As compared with control, a smaller number of GFP^+ cells were observed after *akt* knockout and most of them were PDGFRβ positive (Figure 4(c)). This finding suggested that despite its crucial roles, Akt might not be indispensable in PDGFRβ-mediated cell motility as compared with Src. At 4 weeks postoperatively, the healing effects of different treatments for bone defects were compared. As shown in Supplemental Figure 3, bony development was advanced within control group, as the implants were surrounded by chondrocyte, osteoblast-like cells and filled with livable osteocytes. In the other groups, no viable osteocytes were found in lacunas within bone pieces, and implants were poorly embedded by osteogenesis-related cells. These findings indicated the roles of PDGFRβ, Src, and Akt in the development of bone grafts, thus indirectly providing support for their relevance in motility of host osteoprogenitors.

3.4. Src Is a Bridge Connection between PDGFRβ and Akt during ECs-Induced MSCs Migration. To reveal the relationship between Src and Akt, migrating MSCs were collected *in vitro*. IEC-CM memorably increased the mRNA expression and phosphorylation of Src in MSCs, which were significantly attenuated by knockout of *pdgfrb*, but not *akt* (Figure 5). Moreover, the mRNA expression and phosphorylation of Akt were elevated by IEC-CM. Notably, knockout of *pdgfrb* or *src* impaired the positive effect of IEC-CM on Akt, although no difference was found in the protein level of total Akt. Based on the findings mentioned above, we concluded that in the inflammatory environment, ECs-induced MSCs migration via the PDGFRβ-Src-Akt pathway.

4. Discussion

For bone repair, angiogenesis and osteogenesis are essential processes taking ECs and MSCs as representative involved cells, respectively. They are closely related as evidence indicates the vicinal spatiotemporal loci between ECs and MSCs during bone development and regeneration [3]. Indeed, many perivascular cells exhibit characteristics of mesenchymal progenitors and possess multilineage differentiation potential [19]. Lineage tracing studies suggest that Nestin expressing cells on arteries represent early mesenchymal stem and progenitor cells, with the potential to generate bone lineage cells [20]. In this context, various types of EC-MSC coculture experiments have been conducted to investigate the mechanism and impact of their crosstalk, especially in the development of bone substitutes. In general, findings are positive as MSCs promote ECs-mediated angiogenesis and ECs may regulate the migration and differentiation of MSCs [21]. Accordingly, their coculture has been widely employed to ameliorate repairing efficacy via forming vasculature and inducing vessel ingrowth prior to and after bone grafting, respectively [6]. Nevertheless, unlike the impact of MSCs on ECs, which has been widely described, less is known on the inverse effects. Besides, most of



(a)

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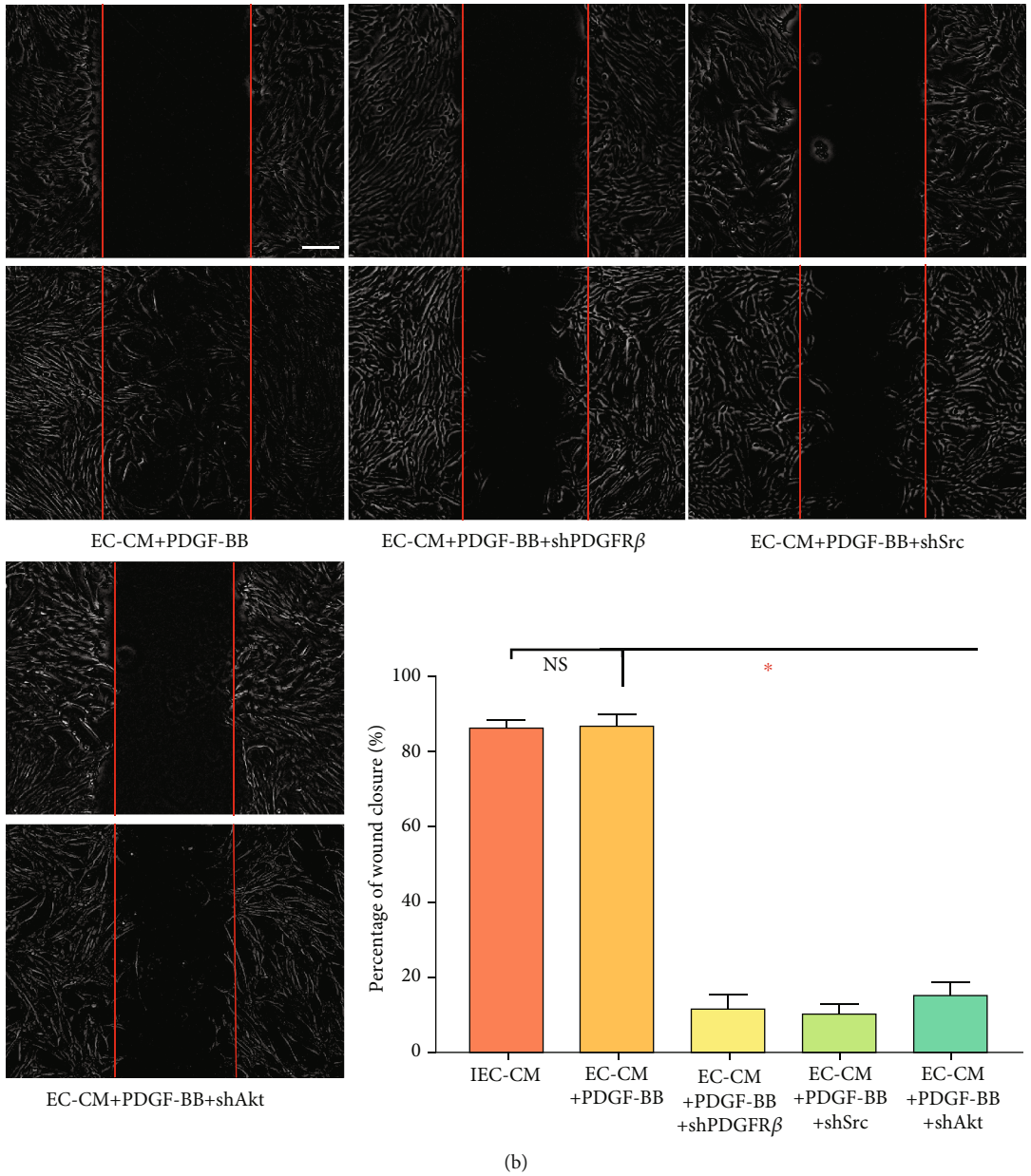
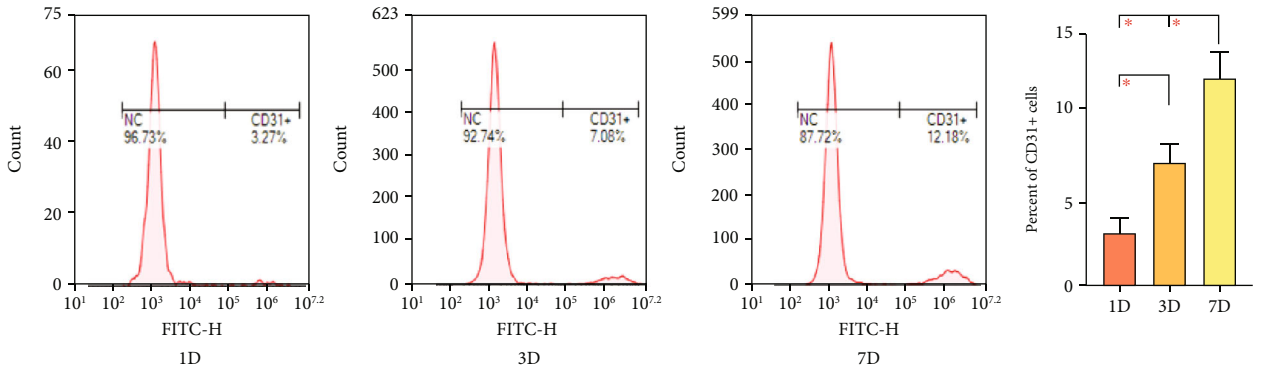


Figure 3: Src and Akt functioned downstream of PDGFRβ. (a) Representative images of migrated hBMSCs in Transwell culture systems. The quantification of migrated cells was shown as a bar graph. Data were compared with the group of IEC-CM from Figure 1. Scale bar, 50 μm. $*P < 0.05$. (b) Representative images of wound healing assays. The rate of scratch wound closure was shown as a bar graph. Data were compared with the group of IEC-CM from Figure 1. Scale bar, 200 μm. $*P < 0.05$. IEC-CM: conditioned media of endothelial cells in the context of inflammatory microenvironment; shPDGFRβ: short hairpin RNA targeting *pdgfrb*; shSrc: shRNA targeting *src*; shAkt: shRNA targeting *akt*.

in vitro studies on their crosstalk are performed under normal conditions [9]. However, the influence of cell crosstalk *in vivo* may be entirely different due to the intricate internal environment. Upon bone injury, local inflammatory responses are incited to form a microenvironment rife with bioactive cellular and molecular components [22]. Consequently, angiogenic or osteogenic cells are educated to change secretome and tropism [14]. On one side, the stimulation of angiogenic cells enables and impulses angiogenesis. Other than nutrient supply and metabolite exchange, homing of osteoprogenitors, as well as the fateful event of bone repair, depends heavily on local vascularization status. On the other side, the inflammatory microenvironment modulates the secretome of MSCs and fosters congeneric recruitment [14]. Therefore, the inflammatory situation cannot be ignored while studying EC-MSC crosstalk. In this study, we introduced a mimicking inflammatory microenvironment *in vitro* to investigate cell motility. IL-1β, IL-6, and TNF-a are representative pro-inflammatory cytokines with peak levels in the early inflammatory phase of bone healing and play crucial roles in bone reconstruction by triggering highly complicated biological cascades [23]. Compared with



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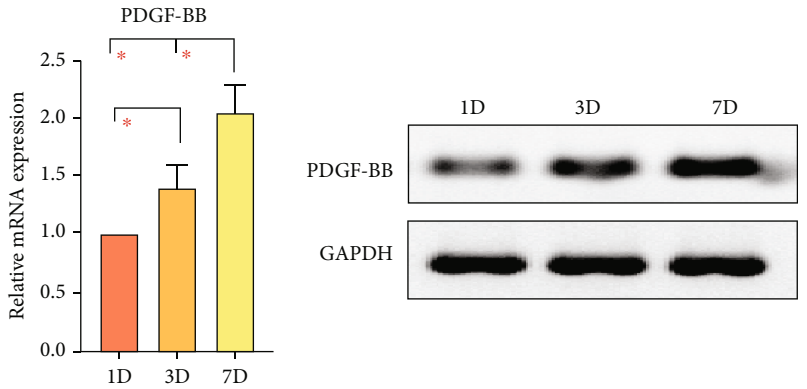

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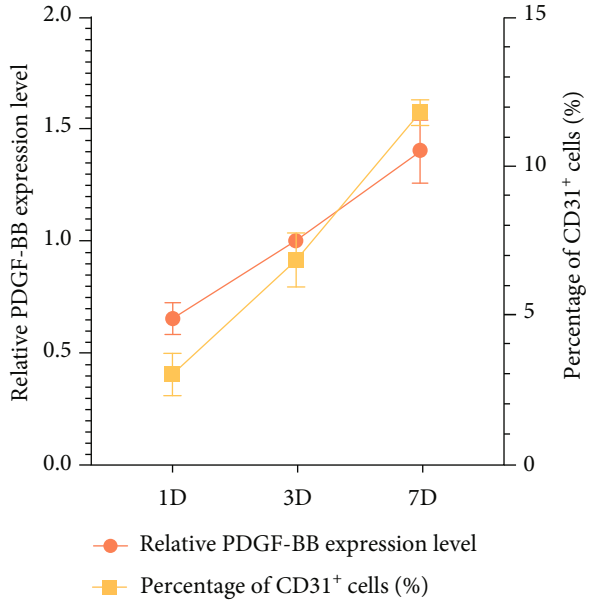

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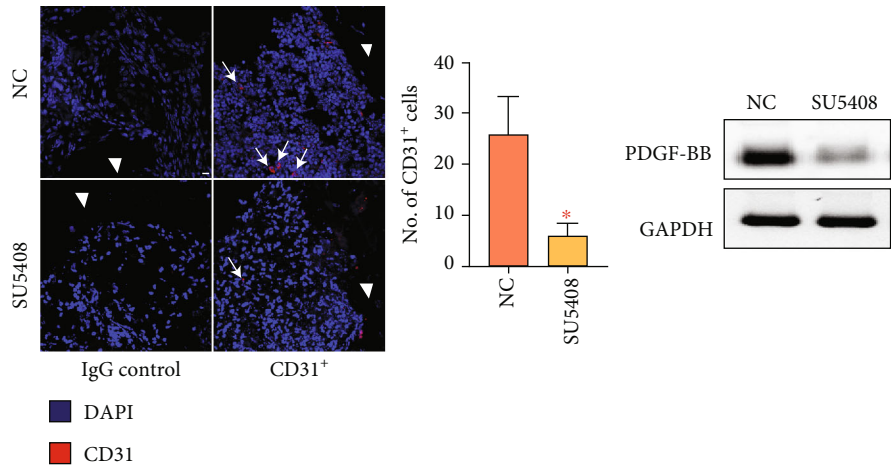

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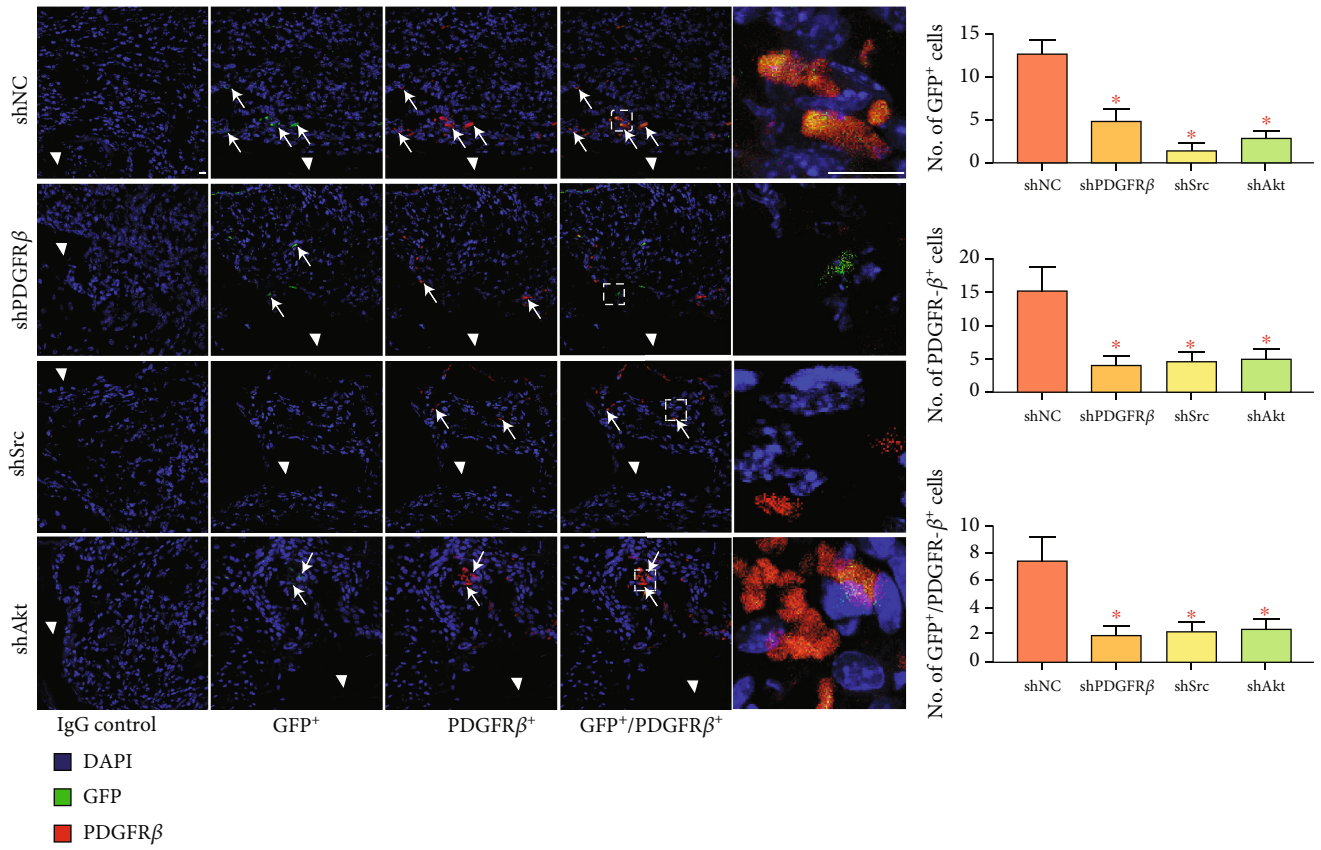

(e)

Figure 4: Data of *in vivo* experiments. (a) Fluorescence-activated cell sorting of $CD31^+$ cells within implants at postoperative days 1, 3, and 7. The percent of $CD31^+$ cells migrated cells was shown as a bar graph. (b) Gene and protein expression of PDGF-BB in sorted $CD31^+$ cells. Data were shown as bar graphs. (c) A positive correlation lay between the ratio of $CD31^+$cells and the protein level of PDGF-BB (Pearson's correlation coefficient $R = 0.926$, $P < 0.05$). (d) Representative images of infiltrated $CD31^+$ cells and PDGF-BB levels within implants. The quantitative comparison was detailed as bar graphs ($n = 5$). (e) Representative images of homed MSCs within implants. The quantitative comparisons were detailed as bar graphs ($n = 5$). Scale bars, 10 μm. $*P < 0.05$. Triangles, implants. Arrows, staining positive cells. shPDGFRβ: short hairpin RNA targeting *pdgfrb*; shSrc: shRNA targeting *src*; shAkt: shRNA targeting *akt*.

common ECs inflammation models, which are usually induced by lipopolysaccharide, the present inflammatory microenvironment is more biomimetic since ECs are exposed during the early stage and cell apoptosis caused by LPS can be avoided effectively [12]. Based on this model, we echoed the concept that ECs could induce MSCs migration physiologically. Under an inflammatory microenvironment, ECs showed a more intensive chemotactic effect on

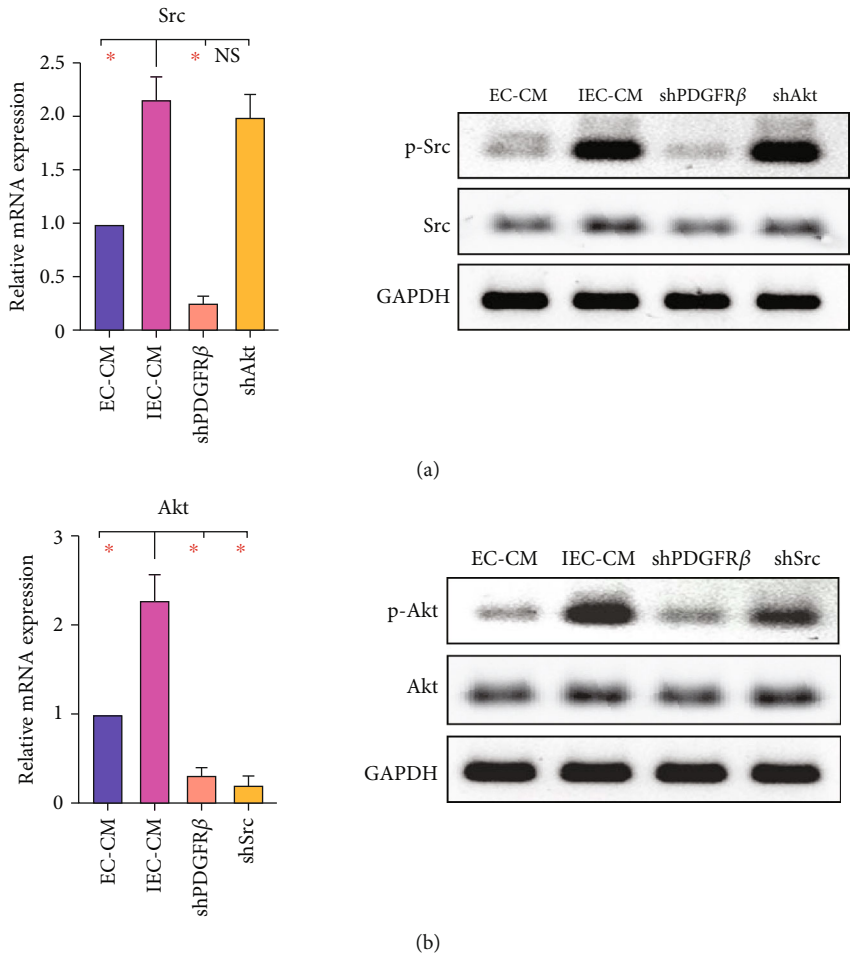

Figure 5: Src bridged connection between PDGFRβ and Akt during ECs-induced MSCs migration. (a) Gene and protein expression of Src in migrating hBMSCs. (b) Gene and protein expression of Akt in migrating hBMSCs. EC-CM: conditioned media of ECs; IEC-CM: conditioned media of ECs in the context of inflammatory microenvironment; shPDGFRβ: short hairpin RNA targeting *pdgfrb*; shSrc: shRNA targeting *src*; shAkt: shRNA targeting *akt*. $*P < 0.05$.

MSCs. This phenomenon is readily comprehensible considering that when new blood vessels grow into the local inflammatory loci, ECs release abundant chemokines to guide vessel-associated MSCs entering to form sheets of osteoblasts, which then secrete osteoid to fabricate bones as oriented by the invading vessels.

Among the multiple chemokines of ECs, the PDGF family has been recognized with significance in the angio-osteogenic coupling [13]. PDGF consists of four polypeptides A, B, C, and D, which assemble into disulfide-linked homodimers or heterodimers (PDGF-AA, -BB, -CC, -DD, or -AB). Thereinto, PDGF-BB is the only dimer with high affinity to all known receptor isoforms and has drawn extensive attention. During bone repair, PDGF-BB plays an integral role in coordinating and linking ECs, MSCs, the extracellular matrix, and signaling pathways [24]. More accurately, PDGF-BB/PDGFRβ constitutes the principal pathway responsible for the activation and function of MSCs, the proliferation and migration of pericytes, and the development of vasculature and new bones. PDGF-BB is mainly secreted from ECs, preosteoclasts, and platelets and supports migration, proliferation, and differentiation of various bone marrow-derived mesenchymal cells to promote angiogenesis and osteogenesis [13]. Herein, we reported that the inflammatory microenvironment forced ECs to secrete an exponential amount of PDGF-BB. Moreover, the promigratory effect of ECs was visibly inhibited by blockade of PDGFRβ. These results collectively confirmed the authority of PDGF-BB/PDGFRβ in osteoprogenitor homing, a pivotal event in the early inflammatory stage of bone repair.

Various signal molecules downstream of PDGF-BB/PDGFRβ have been identified with influences in different disease models. During bone modeling and remodeling, the binding of PDGF-BB and PDGFRβ triggered PI3K/Akt and MAPK signaling cascades, promoting the formation of Type-H vessels and the migration of osteoprogenitors [25]. Also, PI3K and MAPK were requisite in PDGF-BB-mediated MSC motility toward glioma [26]. Previous studies on osteogenic MC3T3-E1 cells showed that the mitogenic response stimulated by PDGF-BB was dependent on extracellular signal-regulated kinase (Erk) and JNK, whereas the migratory response involved MAPK and JNK [27]. JNK was further verified with significance in PDGF-induced

proliferation and migration of MSCs [28]. Endothelial progenitor cells were reported to facilitate viability and nerve regenerative ability of MSCs via PDGF-BB/PDGFRβ and downstream PI3K/Akt and MEK/Erk pathways. Besides, Src played key roles in the migration of metanephric mesenchymal cells toward PDGF-BB [29]. This study adopted a set of pathway inhibitors to screen the predominant signal molecules involved. We found that Src and Akt were the main effectors downstream of PDGF-BB/PDGFRβ during MSC migration toward ECs in the inflammatory microenvironment. Meanwhile, homing of MSCs to bone defects was significantly impaired when *pdgfrb*, *src*, or *akt* was knocked down. Conversely, the results denied the implication of JNK, MEK, and MAPK. With regard to the difference, there were two aspects of conceivable interpretations. One was the extensive regulatory roles of PDGF-BB/PDGFRβ in cell behaviors: viability, proliferation, differentiation, apoptosis, migration, and communication [24]. Another reason lay in the distinct cell and disease types among the currently available literature [30]. Nevertheless, the concurrent involvement of Src and Akt in PDGF-BB-mediated MSCs migration in the inflammatory microenvironment was verified for the first time.

Although Src and Akt link a variety of cell receptors to elicit impacts, their relationship in terms of cell motility remains confused. Most opinions support the upper position of Src. For example, Src acts upstream of Akt in the neural cell adhesion molecule-regulated proliferation, apoptosis, autophagy, migration, and epithelial-to-mesenchymal transition of human melanoma cells [31]. During ASAP1-regulated osteogenic differentiation of MSCs, Src and Akt were implicated and Akt served as the downstream effector. Yet, there is evidence demonstrating the regulatory effect of Akt on Src [32]. As with MSCs, the influence of Src or Akt has been generally accepted; however, little is known on their interaction with regard to motility regulation. Limited evidence suggests that Src may regulate the proliferation and osteogenic differentiation of MSCs via Akt [33]. Here, we showed that shRNA targeting Src downregulated the mRNA expression and phosphorylation of Akt in MSCs. Conversely, Akt shRNA had no significant effect on Src. Thus, ECs recruited MSCs in the inflammatory environment through PDGF-BB/PDGFRβ and its downstream Src-Akt signaling pathway.

There are some limitations in the present study. First, we failed to establish the bone defect models in mice where PDGF-BB was conditionally knocked out in ECs. The death rate was excessive after femoral defects were made with the approach detailed above. This indirectly supported the vital roles of PDGF-BB in sustaining ECs function. As a compromise, MSCs with silenced gene expression were injected back via tail vein, making the confidence level a little weak. Second, the secretory profile of ECs in the inflammatory environment was not fully plotted and there may be other biologics and signaling pathways affecting MSCs migration. Finally, the functional mechanism between and following Src and Akt was not assessed in depth. Further experiments based on proteomics and genomics are needed to gain more insights.

In conclusion, to our knowledge, this study first reveals the role of PDGF-BB/PDGFRβ, as well as downstream Src and Akt signaling, in promoting MSC migration toward ECs in the inflammatory microenvironment. Understanding the functional interplay between ECs and MSCs is practically significant with regard to monitoring processes implicated in bone development after implantation and providing clues for efficacy promotion.

Data Availability

The FACs, transwell assay, wound healing assay, immunofluorescent staining, RT-PCR, and western blot data used to support this study's findings are included in the article. The western blot and Elisa data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

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

Authors' Contributions

Sihao He and Tianyong Hou contributed equally to this work and share first authorship.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1. Scheme illustration for *in vivo* experiments. Femoral critical-sized bone defects were created in C57 mice and DBM were implanted. Implants harvested at postoperative day 1, 3, and 7 were subjected to fluorescence-activated cell sorting (FACs), RT-PCR, and western blot. At 7 days, wild GFP^+ mBMSCs or cells intervened by shRNA were injected via tail vein every 2 days. Implants harvested at 10 days and 4 weeks were subjected to immunofluorescence and HE&Masson staining, respectively. Supplementary Figure 2. (A) ELISA results. (B) Interference efficiency of shRNA targeting *pdgfb* in HUVECs. (C) Interference efficiencies of shRNA targeting *pdgfrb*, *src*, and *akt* in hBMSCs. (D) Interference efficiencies of shRNA targeting *pdgfrb*, *src*, and *akt* in mBMSCs. Supplementary Figure 3. Representative images of H&E staining and Masson staining. At 4 weeks postoperatively, bony development was advanced within control group, as the implants were surrounded by chondrocyte, osteoblast-like cells, and filled with livable osteocytes. In the other groups, no viable osteocytes were found in lacunas within bone pieces, and implants were poorly embedded by osteogenesis-related cells. Scale bars, 500 μm. *(Supplementary Materials)*

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Review Article

Epigenetic Regulation of Methylation in Determining the Fate of Dental Mesenchymal Stem Cells

Hui Zhang[1], **Hong Fu**[1], **Hongzhi Fang**[2], **Qing Deng**[1], **Hao Huang**[3], **Dingyu Hou**[3], **Miaomiao Wang**[4], **Quanzhou Yao**[1], **Qiqi Si**[5], **Rui Chen**[1], **Linke Li**[1], **Jie Weng**[3], **Tailin Guo**[1] and **Mengyuan Wang**[2,6]

[1]*College of Medicine, Southwest Jiaotong University, Chengdu, Sichuan, China 610031*
[2]*Department of stomatology, The Third People's Hospital of Chengdu, The Affiliated Hospital of Southwest Jiaotong University, Chengdu, Sichuan, China 610000*
[3]*College of Materials Science and Engineering, Southwest Jiaotong University, Chengdu, Sichuan, China 610031*
[4]*College of Analysis and Testing Center, Southwest Jiaotong University, Chengdu, Sichuan, China 610031*
[5]*College of Life Science, Southwest Jiaotong University, Chengdu, Sichuan, China 610031*
[6]*The Center of Obesity and Metabolic Diseases, Department of General Surgery, The Third People's Hospital of Chengdu & The Affiliated Hospital of Southwest Jiaotong University, No. 19 Yangshi Road, Chengdu, Sichuan, China 610031*

Correspondence should be addressed to Jie Weng; jweng@swjtu.cn, Tailin Guo; tlguo@home.swjtu.edu.cn, and Mengyuan Wang; wangmengyuan@swjtu.edu.cn

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Dental mesenchymal stem cells (DMSCs) are crucial in tooth development and periodontal health, and their multipotential differentiation and self-renewal ability play a critical role in tissue engineering and regenerative medicine. Methylation modifications could promote the appropriate biological behavior by postsynthetic modification of DNA or protein and make the organism adapt to developmental and environmental prompts by regulating gene expression without changing the DNA sequence. Methylation modifications involved in DMSC fate include DNA methylation, RNA methylation, and histone modifications, which have been proven to exert a significant effect on the regulation of the fate of DMSCs, such as proliferation, self-renewal, and differentiation potential. Understanding the regulation of methylation modifications on the behavior and the immunoinflammatory responses involved in DMSCs contributes to further study of the mechanism of methylation on tissue regeneration and inflammation. In this review, we briefly summarize the key functions of histone methylation, RNA methylation, and DNA methylation in the differentiation potential and self-renewal of DMSCs as well as the opportunities and challenges for their application in tissue regeneration and disease therapy.

1. Background

Dental mesenchymal stem cells (DMSCs) are multipotent progenitor cells with multilineage differentiation and self-renewal capability [1]. Recently, DMSCs have been obtained from periodontal, periapical, and pulpal tissue from permanent teeth [2]. DMSCs, a group of multipotent MSCs, contain dental follicle stem cells (DFSCs) [3], stem cells from alveolar bone (ABMSCs) [4], periodontal ligament stem cells (PDLSCs) [5], dental pulp stem cells (DPSCs) [6], stem cells from apical papillae (SCAPs) [7], stem cells from exfoliated deciduous teeth (SHED) [2], and stem cells from gingival tissue (GMSCs) (Figure 1) [8, 9]. DMSCs can differentiate into odontoblasts, chondrocytes, osteoblasts, adipocytes, neurons, and so on [10]. The excellent properties of DMSC make them play a critical role in tissue engineering and regenerative medicine [10–13].

Epigenetic regulation changes gene expression without altering the DNA sequence, meaning altering the gene expression potential stably during cell proliferation and

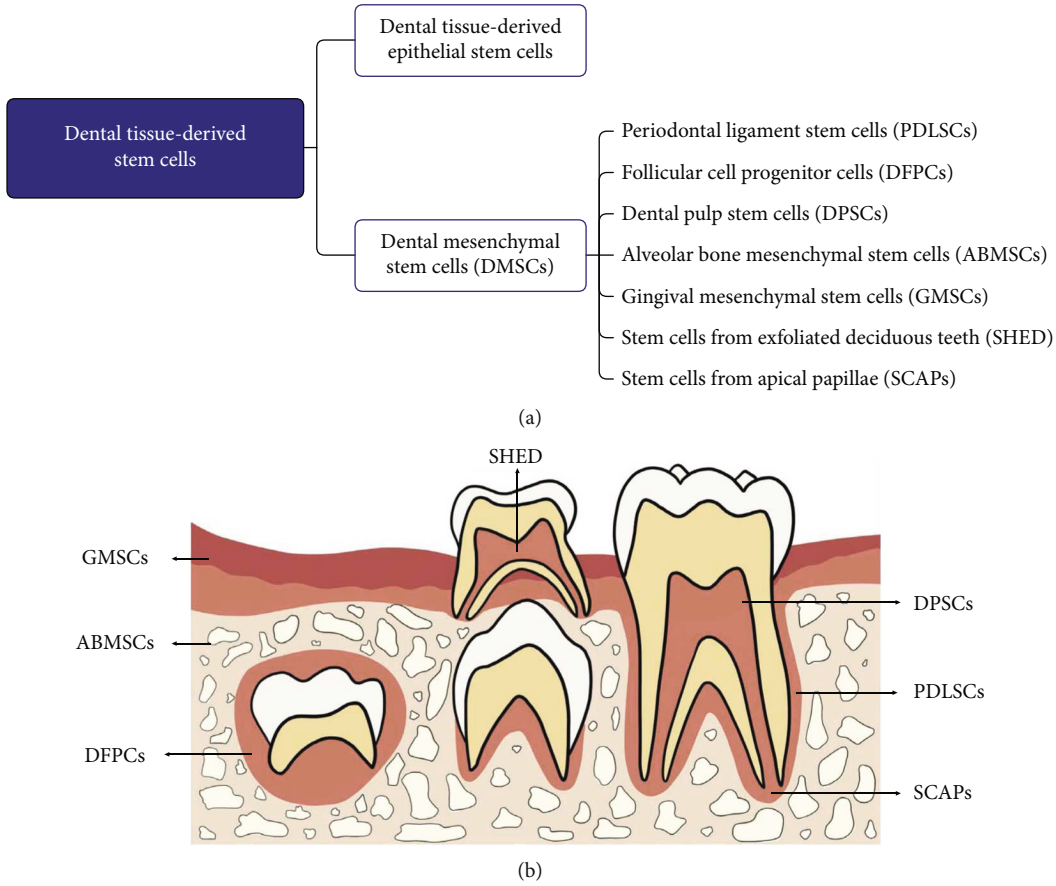

Figure 1: The different populations of dental tissue-derived stem cells and their distribution. Dental tissue-derived stem cells include dental mesenchymal stem cells and dental tissue-derived epithelial stem cells. DPSCs: dental pulp-derived stem cells; DFSCs: dental follicle stem cells; SCAPs: stem cells from apical papilla; PDLSCs: periodontal ligament stem cells; SHED: stem cells from exfoliated deciduous teeth GMSCs: stem cells from gingival tissue; ABMSCs: stem cells from the alveolar bone.

differentiation [14]. Epigenetic mechanisms can promote appropriate biological behavior by postsynthetic modification of DNA or protein, making the organism adapt to developmental and environmental prompts by regulating gene expression [15, 16]. The main mechanisms of epigenetic regulation include methylation, ubiquitination, acetylation, and chromatin remodeling, which have been proven to exert a significant effect on the behavior of DMSCs, such as proliferation, self-renewal and differentiation potential [16]. Recently, there have been quantitative studies on the epigenetic underline of the biological and pathological processes in embryogenesis, development, and diseases. One important research topic is the effect of methyltransferase on DMSC fate, which includes DNA methylation, RNA methylation, and histone modifications [15, 17–19]. For example, both the histone demethylase lysine (K)-specific demethylase 6B (KDM6B) and lysine demethylase 2A (KDM2A) [20] participate in the osteogenic differentiation of DMSCs [21]. The histone demethylase KDM6B specifically demethylates dimethylation/trimethylation on lysine 27 of histone 3 (H3K27me2/3) and reactivates bone morphogenetic protein 2 (BMP2), which regulates osteogenic differentiation of DMSCs and plays a critical role in dental regeneration [22]. The silencing of KDM2A increases the H3K36me2 levels in the promoter of SFR2, which enhances the odontoblast differentiation potential of SCAPs [21]. 5-Aza-2′-deoxycytidine (5-Aza) is a DNA methyltransferase inhibitor that enhances the odontogenic differentiation of PDLSCs in DMSCs by decreasing the proliferation rate [4]. It was reported that DNA/histone methylation can regulate the osteogenic differentiation and proliferation of DPSCs [23].

In this review, we briefly summarize the key functions of histone methylation, RNA methylation, and DNA methylation in the differentiation potential and self-renewal of DMSCs as well as the opportunities and challenges for their application in tissue regeneration and disease therapy.

2. Characteristics and Clinical Potential of DMSCs

DMSCs have excellent properties, including self-renewal potency and multipotent differentiation capacity, which provide a new pathway for regenerative medicine [24]. Similar

to stem cells (SCs), DMSCs are identified via cell surface markers, such as CD13, CD29, CD73, CD44, CD90, and CD105. Meanwhile, DMSCs negatively express hematopoietic markers, including CD14, CD19, CD34, CD45, CD11b, and HLR [25].

Among these DMSCs, DPSCs have attracted increasing attention in tissue regeneration due to their clonogenic efficiency and easy availability [26–29]. Moreover, DPSCs have the capacity for odontogenic and osteogenic differentiation to form dentin and bone tissues. PDLSCs exhibit multilineage differentiation potential and are isolated from human periodontal ligament. PDLSCs can be effectively applied in bone defect repair by modulating the immune microenvironment of the dental complex [30, 31]. A series of studies have demonstrated the multilineage differentiation potential and immunosuppressive property of DFSCs, and their unific characteristics make them applicable to the repair of periodontal defects and repression of inflammation in chronic inflammatory disease [32]. SCAPs can differentiate into osteoblasts, nerve cells, and adipocytes and exhibit immunosuppressive features [33]. SHED can regulate T cells and repress the function of T helper 17 cells to achieve immunomodulatory functions [34]. ABMSCs can regenerate new periodontium tissues and alveolar bone [35]. GMSCs can repair tongue muscle, mandibular, and calvaria defects as well as improve cementum, periodontal ligament, and alveolar bone regeneration [36, 37]. MSCs derived from the tooth germ (TGSCs) show excellent potential in osteogenic differentiation. In addition, TGSCs can differentiate into osteogenic, chondrogenic, neurogenic, and adipogenic cells [38]. TGSCs could be used in gingival tissue regeneration and therapy because of their easy accessibility and excellent differentiation potential [32, 39].

Currently, DMSCs can be applied to the therapy of oral diseases. DMSCs can reduce the inflammatory response by inhibiting the release of inflammatory cytokines to treat periodontal disease by promoting alveolar bone regeneration [1, 40]. Clinical studies found that DMSCs were able to regenerate compact substances of human mandibles, suggesting that DMSCs can be an attractive source of autologous transplantation for regenerative treatment [41, 42]. For example, PDLSCs with bone grafting material were transplanted into 16 defective teeth of three periodontitis patients, and all probing depths were reduced [43]. In bone tissue engineering, PDLSCs combined with tissue engineering scaffolds could regenerate alveolar bone in 30 periodontitis patients without significant adverse effects [44]. Autologous PDLSC niches were transplanted in 14 patients, and the probing pocket depth was reduced [45]. DPSCs combined with the scaffold material were implanted into the defect area of root furcation in two patients, and the results demonstrated that the periodontal defects were repaired [46]. The transplantation of DPSCs in five pulpitis patients promoted dentin formation safely and effectively [47]. Moreover, when DPSCs were mixed with support material and imputed into the bone defect of periodontitis patients, the bone defect was repaired, bone mineral density was increased, and the periodontal pocket depth was decreased in patients [48, 49].

3. Methylation Modifications of Epigenetics

Methylation modifications are the most common mechanism in complicated epigenetic processes and can regulate genes by changing the state of chromatin without altering the DNA sequence in cells [50], thus playing a vital role in gene expression, protein function, and RNA processing. Furthermore, methylation modifications include histone methylation, DNA methylation, and RNA methylation [51], which are dynamic processes regulated by methyltransferases and demethylases of histones, DNA, and RNA (Figure 2). These enzymes could control the fate of stem cells by mediating their pluripotency or differentiation [9, 52–56].

3.1. DNA Methylation. DNA methylation is a specific epigenetic mechanism that regulates gene expression and SC functions [57–60]. DNA methylation refers to the symmetrical addition of a methyl group on the 5-position of cytosine. This process is catalyzed by a group of enzymes, DNA methyltransferases (DNMTs), including DNMT1, DNMT3 L, DNMT3B, and DNMT3A [17, 61–66]. DNMTs at the cytosine residue at the 5-position in CpG dinucleotides transfer methyl groups of SAM (S-adenosylmethionine) to SAH (S-adenosylhomocysteine) and generate 5-methylcytosine (5-mC) [67]. DNA 5mC was discovered in 1948 and marked the prolusion of the consecutive research in epigenetic modification [66]. As one of the most common modification sites in eukaryotes [14, 68–70], it represses the binding of RNA polymerase and recruits binding proteins and thereby typically acts on epigenetic silencing of gene expression [18, 71]. DNA methylation status is stabilized via DNMTs and DNA demethylases. DNA demethylation is also vital during cellular proliferation and differentiation. 5-mC is converted to 5-hydroxy methylcytosine (5-hmC) via an oxidation reaction. This process is the first pathway of DNA demethylases and is mediated by the Ten–eleven translocation (TET) family, including TET1, TET2, and TET3 [61, 72]. Furthermore, thymine DNA glycosylase (TDG) could convert 5-hmC back to complete DNA demethylation and make the cytosine totally unmethylated, which is associated with the modification of G/T mismatches in DNA repair [17, 72] (Figure 3). Thus, DNA methylation plays a crucial role in the fate of SCs by gene regulation [59, 72].

3.2. Histone Methylation. DNA twines on histone proteins, including two dimers, H3/H4 and H2A/H2B, all of which compose a globular histone octamer and form the basic units of chromatin as nucleosomes [73]. The special structure of histones in eukaryotes exhibits a diversity of histone modifications, such as methylation, acetylation, phosphorylation, and ADP ribosylation [74]. Histone methylation induces alterations in chromatin structure by adjusting the density of nucleosomes, which regulates gene transcription [73] (Figure 4). Histone methylation mainly occurs at arginine and lysine residues of the tail in H3/4 and is mediated by protein lysine methyltransferases (PKMTs) and protein arginine methyltransferases (PRMTs) [74]. The lysine methylation sites, including histone H3 at lysine 4 (H3K4), H3K79, H3K36, H3K27, and H3K9, and trimethylation sites

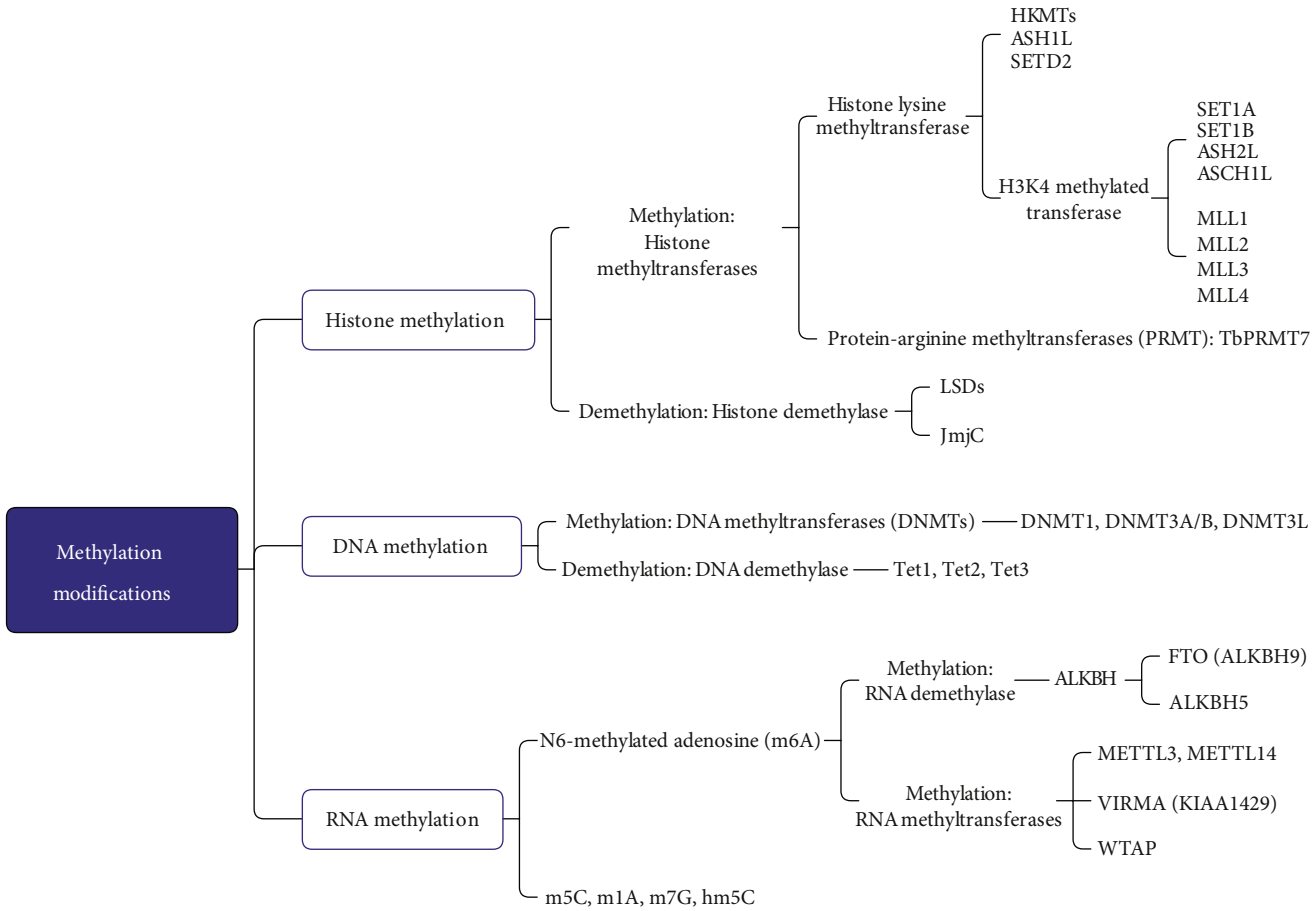

Figure 2: Methylation modifications' classification and main enzymes involved in methylation. Methylation modifications include DNA methylation, histone methylation, and RNA methylation. The enzymes include methyltransferase and demethylase.

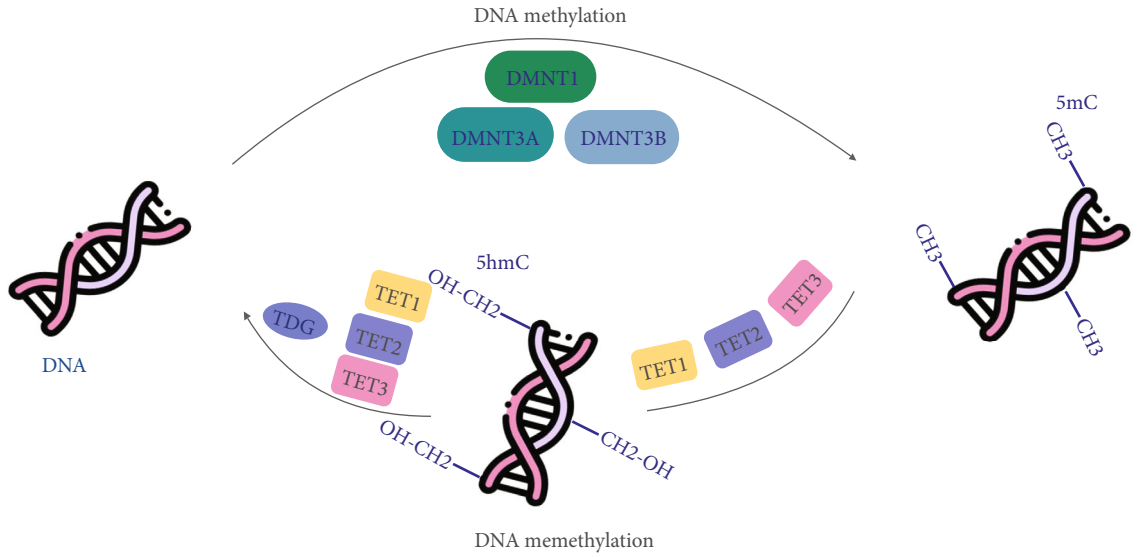

Figure 3: Schematic representation of DNA methylation patterning. DNA methylation is exerted by DNA methyltransferases (DNMTs), including DNMT1, DNMT3L, DNMT3B, and DNMT3A. DNMTs at the cytosine residue at the 5-position in CpG dinucleotides transfer methyl groups and generate 5-methylcytosine (5-mC). DNA demethylation involves the successive oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC) by Ten–eleven translocation (TET) family, including TET1, TET2, and TET3. Thymine DNA glycosylase (TDG) could convert 5-hmC back to make the cytosine totally unmethylated.

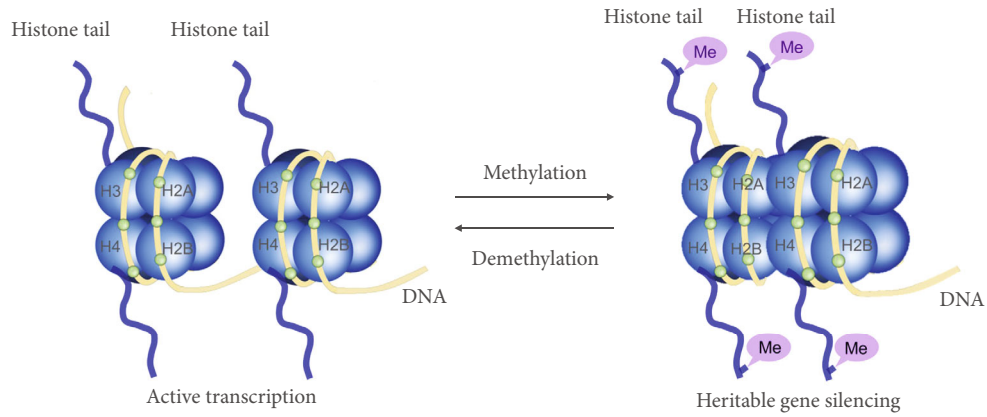

Figure 4: Schematic representation of histone methylation and demethylation. DNA twines on histone proteins, including two dimers, H3/H4 and H2A/H2B, all of which compose a globular histone octamer and form the basic units of chromatin as nucleosomes. Histone methylation mainly occurs at arginine and lysine residues of the tail in H3/4 and is mediated by methyltransferases and histone demethylation is regulated by demethylases. Histone methylation can affect the spatial structure of chromatin by affecting the structure of nucleosomes and thus regulating the expression activity of genes. The nucleosome structure usually becomes crowded by adding methyl group (Me) from arginine and lysine residues of the tail, which making it difficult for gene segments to be transcribed, so gene expression is silenced. In contrast, the demethylation of histone usually could induce the open histone structure by removing methyl group (Me) from arginine and lysine residues of the tail, which expose the transcription factor binding sites and regulates the transcriptional activation of genes.

on lysine 4 of histone 3 (H3K4me3), lysine 27 of histone 3 (H3K27me3), and lysine 9 of histone 3 (H3K9me3) have been widely studied for the modification of genes [73, 75–77]. For instance, PRMTs catalyze arginine methylation. The yeast Dot1 and the mammalian homolog telomeric silencing 1 like (DOT1L) catalyze lysine methylation [78, 79]. Histone demethylases mainly contain the family of dioxygenase Jumonji-C (JmjC) domain proteins and the amine oxidases family of non-JmjC proteins, such as KDM6B and lysine-specific demethylase1 (LSD1) [20]. For example, KDM6B, a member of the oxidase family in the JmjC domain, can downregulate insulin-like growth factor binding protein 5 (IGFBP5) to enhance periodontal tissue regeneration by MSCs [80, 81]. LSD1 can reverse the methylation of H3K4, which may repress gene transcription [20, 76, 82]. Histone methylation has been widely studied and affects the fate of SCs [83, 84], such as cancer stem cells, adult tissue stem cells, and embryonic stem cells [19, 78, 83]. Additionally, histone methylation has the ability to regulate ESC differentiation and maintain the regeneration of neural stem cells and muscle stem cells [85]. They can also promote liver regeneration in animal experiments [86].

3.3. RNA Methylation. RNA methylation refers to the process of adding a methyl group to the methyl adenine of RNA [87]. RNA methylation includes N1-methyladenosine (m1A), N6-methyladenosine (m6A), 7-methyl guanosine (m7G), and 5-methylcytosineylation (m5C) modification of mRNA in eukaryotes [88–90]. As one of the most general RNA methylations, m6A RNA methylation refers to methylation of the adenosine (A) base at the nitrogen-6 site [87], and this has attracted more attention in recent years. RNA methylation is a reversible and posttranslational modification of RNA by catalysis of methyltransferase and demethylase [91]. m6A RNA methyltransferases catalyze RNA methylation and are known as "writers," including methyltransferase-like protein 3 (METTL3), METTL14, and WTAP [92, 93]. The m6A RNA demethylase is known as an "eraser," containing obesity-associated protein (FTO) and fat mass ALKB homolog 5 (ALKBH5), which regulates RNA demethylation [87, 89]. Readers include the YTH domain family (YTHDFs and YTHDCs) and IGF2BP1/2/3 family, which can directly bind to m6A to mediate downstream processes including mRNA export and mRNA translation [89–93] (Figure 5). m6A RNA methylation plays a critical role in biological processes by affecting gene translation, the DNA damage response, autophagy, stem cell proliferation, and fat formation [87, 89, 94–97]. For example, METTL3, as the most well-studied subunit of the m6A "writers," could slow down the occurrence of chronic obstructive pulmonary disease and might promote tumor formation, migration, and invasion [98–100]. In addition, ALKBH5 has high expression in the embryonic stage and glioblastoma stem-like cells (GSCs), revealing that ALKBH5 may play an indispensable role in brain development and GSC proliferation [101–103]. FTO is associated with human diseases, including obesity, type 2 diabetes, coronary heart disease, and cancer [90, 104, 105].

4. Regulation of DMSCs by Methylation

Methylation has been proven to play a critical role in DMSCs, including PDLSCs, DFSCs, DPSCs, and SCAPs [4]. We briefly summarize some recent studies on methylation modifications and the clinical application potential of DMSCs.

4.1. PDLSCs. PDLSCs can be isolated from the periodontal ligament [106], and they can differentiate into alveolar bone, peripheral nerves, blood vessels, adipocytes, hepatocytes,

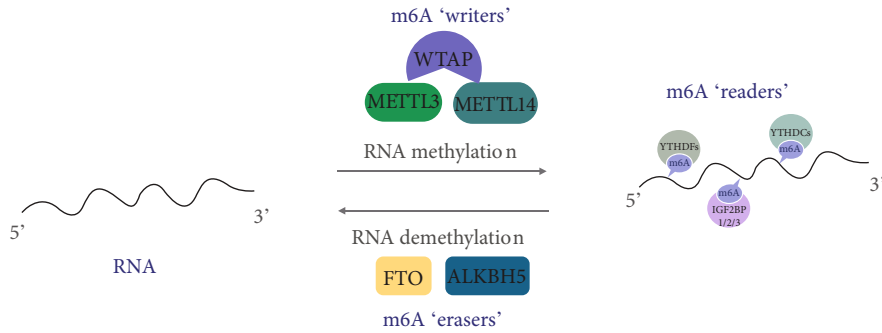

Figure 5: The dynamic and reversible processes of m6A methylation. m6A RNA methylation is mainly regulated by its "writers," "erasers," and "readers." Writers refer to the m6A methylase complex including METTL3, METTL14, and WTAP. Erasers are m6A demethylases involving FTO and ALKBH5. Readers include the YTH domain family (YTHDFs and YTHDCs) and IGF2BP1/2/3 family. "Writers" deposit m6A methylation on RNAs, while "erasers" remove the m6A marks. "Readers" can directly bind to m6A to mediate downstream processes including mRNA export and mRNA translation.

and osteoblasts under specific conditions [21, 107]. PDLSCs have a positive effect on alveolar bone formation, which would rescue the loss of alveolar bone in periodontitis [108]. Furthermore, PDLSCs are used for repair of periodontal tissues and treatment of cartilage diseases in the regenerative medicine field [109, 110].

4.1.1. Modification of PDLSCs by DNA Methylation. DNA methylation is a key regulatory component of epigenetic modification. Some papers have suggested that DNA methylation can regulate the fate of PDLSCs (Table 1). In the genomic analysis of DNA methylation, the DNA methylation of bone formation-related genes in PDLSCs was different from that in DPSCs and DFPCs. PDLSCs have lower methylation of osteogenic-related genes, such as runt-related transcription factor 2 (RUNX2), osteopontin (OPN), and alkaline phosphatase (ALP), leading to PDLSCs with better bone formation capacity in vivo [111]. Therefore, a better understanding of the DNA methylation of genes in PDLSCs is crucial to regulating osteogenic differentiation of PDLSCs and regeneration of periodontal tissue. For instance, the expression of RUNX2 was enhanced in the coculture of dedifferentiated fat cells (DFATs) with PDLSCs due to the downregulation of RUNX2 DNA methylation, which promoted the osteogenic potential of PDLSCs and DFATs [112]. Furthermore, hypermethylation of RUNX2 inhibits osteogenic differentiation of PDLSCs [113], and RG108 and 5-Aza, as DNMT1 inhibitors, could restore RUNX2 expression and increase the osteogenic potential of PDLSCs by eliminating the upregulated expression of DNMT1 in PDLSCs [114–116]. In addition, the destruction of the periodontium caused by lipopolysaccharide (LPS) leads to hypermethylation of RUNX2 in PDLSCs, which might directly hinder periodontal regeneration [115]. 5-Aza and RG108 may be potential therapies for periodontal diseases by restoring the hypermethylation of RUNX2.

In addition, a high-glucose (HG) environment inhibits the proliferation and differentiation of PDLSCs [117]. Increasing the expression of DNMT3A, DNMT3B, and DNMT1 in an HG environment causes DNA hypermethylation of osteogenic-related genes in PDLSCs, which has an inhibitory effect on the matrix mineralization of PDLSCs. However, 5-Aza reduces the DNA hypermethylation level and recovers the expression of osteogenic marker genes, such as ALP, OCN, OPN, and osterix (OSX, also called Sp7), thus rescuing matrix mineralization and stimulating osteogenic differentiation of PDLSCs [108]. In addition, tumor necrosis factor α (TNF-α) treatment of PDLSCs in an HG environment could cause PDLSCs to have lower cell viability [118]. The HG environment also inhibits the expression of DNMT1 protein while upregulating tumor necrosis factor-alpha receptor-1 (TNFR-1) due to the hypomethylation of CpG islands within the TNFR-1 gene in PDLSCs. However, SAM could downregulate TNFR-1 by increasing the methylation of TNFR-1 and then downregulating TNF-α and rescuing the cell viability of PDLSCs [118]. Modulation of DNA methylation by SAM or 5-Aza could regulate the viability and differentiation of PDLSCs in HG, which would be a potential treatment for periodontitis [108, 118].

On the other hand, inhibition of Ten–eleven translocation 1 (TET1) and Ten–eleven translocation 2 (TET2) could lead to the downregulation of the osteogenic and adipogenic capacity of PDLSCs, while the proliferation of PDLSCs could be enhanced [119]. In addition, Dickkopf-related protein-1 (DKK-1) exerts a negative effect on the Wnt pathway, thus inhibiting the immunomodulatory capacity of PDLSCs [120]. TET1 and TET2 binding to the DKK-1 promoter could maintain the hypomethylation of DKK-1 in PDLSCs and then inhibit the function of T cells induced by PDLSCs. Therefore, inhibition of TET1 and TET2 could result in hypermethylation of the DKK-1 promoter and significantly inhibit the expression of DKK-1 and in turn enhance the immunomodulatory capacity of PDLSCs [121]. Thus, downregulation of TET1 and TET2 may enhance the effect of PDLSC-mediated immunotherapy [119, 122].

4.1.2. Modification of PDLSCs by Histone Methylation. Histone methylation is widely known to regulate the proliferation and differentiation of PDLSCs (Table 1). For example, downregulating the long noncoding RNA SNHG1 and upregulating Kruppel-like factor 2 (KLF2) both promote

Table 1: Methylation and demethylation in PDLSCs.

Methylated modification	Epigenetic modifiers	Epigenetic marks	Functions
DNA methylation	RG108	DNMT inhibitor	The hypomethylation of RUNX2 promoted osteogenic potential [116].
	5-Aza	DNMT inhibitor	Down-regulating expression of DNMT1, stimulating osteogenic differentiation [108, 113, 115, 117, 118].
	SAM	Methyl-donor	Rescuing the cell viability and increasing the methylation of TNFR-1 [118].
DNA demethylation	TET1/2	DNA demethylases	Enhancing differentiation while inhibiting immune regulation [119–122, 129].
Histone methylation	EZH2	H3K27me3	Inhibiting the osteogenic differentiation [110, 123, 124, 129].
	SETD1B	H3K4me3	Downregulating the release of inflammatory factors from PDLSCs stimulated by LPS [125].
	SETD2	H3k36me3	Promoting the osteogenic differentiation [126].
Histone demethylation	KDM6A	H3K27me3, H3K4me3	Enhancing the osteogenic differentiation [22, 127].
	EPZ-6438	EZH2 inhibitor	Rescuing the chondrogenic potential of PDLSCs by decreased H3K27me3 [22].
	KDM6B	H3K27	Enhancing periodontitis inflammatory response and apoptosis [124, 128].
		H3K4me3	Promoting potential of proliferation, chemotaxis and migration [109].

the osteoblastic differentiation of PDLSCs. SNHG1 inhibits the osteoblastic differentiation of PDLSCs by regulating H3K27me3 of KLF2 through zeste homolog 2 (EZH2) [123]. In addition, stress stimulation resulted in the upregulation of H3K27me3 signaling and the slight downregulation of transcription factor 2 (E2F), which induced transcriptomic changes and impaired the pluripotency of PDLSCs. Meanwhile, overexpression of EZH2 enhances the adipogenic differentiation of PDLSCs while suppressing osteogenic differentiation [110, 123]. Downregulation of EZH2 expression enhances ALP activity in PDLSCs induced by LPS [124]. Therefore, EZH2, as a histone methyltransferase, plays an important role in maintaining the differentiation of PDLSCs. Moreover, the in vitro and in vivo results showed that LPS induced the expression of H3K4me3 on inflammation-related genes. Inhibition of the protein lysine methyltransferase SET domain-containing 1B (SETD1B) led to the downregulation of H3K4me3, which decreased inflammatory genes and promoted osteogenic genes in PDLSCs [125]. In addition, upregulating the specific methyltransferase H3K36me3, known as SET domain-containing protein 2 (SETD2), can promote the osteogenic differentiation of PDLSCs [126]. Furthermore, histone methyltransferases such as EZH2, SETD1B, and SETD2 regulate the osteogenic differentiation of PDLSCs.

Histone methylation can be reversed by histone demethylases, such as lysine-specific demethylase 6A (KDM6A) and KDM6B. For instance, KDM6A promotes the expression of osteogenesis-related genes through the demethylation of H3K27me3 in the promoter region and facilitates the osteogenic differentiation of PDLSCs. Hence, overexpression of KDM6A enhances the osteogenic differentiation of PDLSCs [22, 127]. In addition, the absence of KDM6A elevates the level of H3K27me3 and decreases the expression of trimethylation on lysine 4 of histone 3 (H3K4me3), which ultimately suppresses the chondrogenic potential of PDLSCs [22]. However, treatment of PDLSCs with an inhibitor of EZH2 (EPZ-6438) rescued the impaired chondrogenesis of PDLSCs caused by the loss of KDM6A. Therefore, the regulation of KDM6A demethylation and the application of an EZH2 inhibitor potentially induces MSC-mediated cartilage regeneration in osteoarthritis [22]. Furthermore, Jiang and Jia found that miR-153-3 could inhibit the expression of KDM6A and the osteogenic differentiation of PDLSCs [127]. Therefore, downregulation of miR-153-3 or overexpression of KDM6A promotes the osteogenic differentiation of PDLSCs. These findings provide a new potential therapeutic application for PDLSCs in alveolar bone regeneration.

Moreover, both histone methyltransferases and demethylases regulate the fate of PDLSCs in an inflammatory environment. Knockdown of KDM6B inhibits the expression of RUNX2 and inflammatory factors in PDLSCs stimulated by LPS [124]. In addition, treatment of PDLSCs with LPS causes an increase in H3K27me3 on the promoters of OSX, RUNX2 and IL-1β. Therefore, LPS inhibits the proliferation and osteoblastic differentiation capacity of PDLSCs by increasing H3K27me3 on genes in PDLSCs. Glycoprotein nonmetastatic melanoma protein B (GPNMB) reduces LPS-induced apoptosis of PDLSCs as well as upregulates the expression of KDM6B, which could result in inhibiting the inflammatory response and apoptosis in periodontitis [128]. IGFBP5 can promote cell proliferation, chemotaxis, and migration in PDLSCs [80]. However, the deletion of the PR domain containing 9 (PRDM9) gene can upregulate IGFBP5 by increasing H3K4me3 of the IGFBP5 promoter, which enhances the transcription of IGFBP5 and promotes the proliferation, chemotaxis, and migration of PDLSCs [109]. Therefore, histone methylation has the ability to regulate the fate of PDLSCs via the regulation of different genes, such as RUNX2, GPNMB, and PRDM9.

Table 2: Methylation and demethylation in DPSCs.

Methylated modification	Epigenetic modifiers	Epigenetic mark	Function
DNA methylation	DNMT3A	DNMTs	Regulating the odontogenic differentiation [135].
	DNMT3B	DNMTs	Enhancing the odontogenic differentiation of DPSCs by inhibiting DNMT3B-mediated-methylation of DLX3 [135–137].
	DNMT1	DNMTs	The methylation of KLF4 promoters by DNMT1 inhibited the odontoblast differentiation of DPSCs [138].
	RG108	DNMT inhibitor	Improving the efficiency of odontoblast differentiation [138].
	5-Aza	DNMT inhibitor	Inhibiting inflammation while enhancing myogenic differentiation and odontogenic differentiation [141–144].
DNA demethylation	TET2	DNA demethylases	TET2 knockdown downregulates MyD88 promoter methylation and inhibits LPS-induced inflammatory responses in DPSCs [145].
	TET1	DNA demethylases	Enhancing self-differentiation and odontogenesis [146–148].
Histone methylation		H3K4me3	Enhancing the differentiation [150].
	EZH2	H3K27me3	Reducing H3K27me3 expression increased that of KDM6B in DPSCs under inflammation and inhibition of EZH2 can activate β-catenin transcription and Wnt signaling pathway to promote the osteogenic differentiation [151–153].
Histone demethylation	KDM6B	H3K27me3	Removing H3K27me3 methylation, activating odontogenic transcriptional gene activation and enhancing the odontogenic differentiation [151, 154, 155].
	KDM5A	H3K4me3	Demethylation of H3K4me3 suppresses the dentin differentiation of DPSCs [156].
RNA methylation	METTL3	m6A	Regulating the proliferation, migration, differentiation, root formation, cell senescence, and apoptosis. Knockdown of METTL3 reduces the expression of inflammation-related factors and activation of signaling pathways induced by LPS [158/157, 159/158, 160/159].
RNA demethylation	METTL14	m6A	Expressing in PDLSCs [158/157].
	FTO	m6A	Expressing in PDLSCs [158/157].

4.2. DPSCs. DPSCs were first cultured in vitro by Gronthos et al. in 2000 [6]. DPSCs can differentiate into osteoblasts, cartilage heads, fats, blood vessels, odontoblasts, and so on [130]. Compared with other mesenchymal stem cells, DPSCs show better abilities, such as better odontoblast differentiation and viability. Nevertheless, DPSCs showed lower chondrogenic capacity [131–134]. Consequently, the differentiation ability and clinical application potential of DPSCs in regenerative medicine have attracted extensive attention [27].

4.2.1. Modification of DPSCs by DNA Methylation. Numerous studies have also reported the function and regulation of DNA methylation in DPSCs (Table 2). DNMTs, such as DNMT1, DNMT3A, and DNMT3B, have an important effect on the differentiation of DPSCs [135]. DNA methylation mediated by these DNMTs can modulate odontogenic-related genes and calcium nodule formation. For instance, microRNA-675 (miR-675) regulates the odontogenic differentiation of DPSCs by suppressing DNMT3B-mediated methylation of distal-less homeobox 3 (DLX3) [136]. In addition, overexpression of lncRNA H19 (a classic long noncoding RNA) and miR-675 promotes the odontogenic differentiation potential of DPSCs by downregulating DNMT3B-mediated methylation of the DLX3 gene [137]. The methylation of KLF4 promoters mediated by DNMT1 is a transcription factor that can inhibit the odontoblast differentiation of DPSCs [138]. Kruppel-like factor 4 (KLF4) and SP1 enhance the odontoblastic differentiation of DPSCs as transcription factors [139, 140]. In contrast, RG108 and 5-Aza play a positive role in the differentiation of DPSCs. Inhibition of DNMT1 by RG108 increases KLF4, which improves the efficiency of odontoblast differentiation [138]. Hence, RG108, as a DNMT1 inhibitor, mediates the upregulation of SP1/KLF4 during the odontoblast differentiation of DPSCs. 5-Aza inhibits DNMT1 and BMP activity while promoting muscle-specific transcription factors in DPSCs. 5-Aza enhances the skeletal myogenic differentiation of DPSCs [141]. Furthermore, 5-Aza can promote myogenic differentiation and myogenic protein expression of DPSCs, and it can be used for the therapy of craniofacial muscles [142]. DPSCs with 5-Azax also increase ALP activity and calcified nodule formation, while odontogenic markers, including dentin matrix protein-1 (DMP-1), dentin sialo phosphoprotein (DSPP), RUNX2, DLX5, and OSX, are upregulated [143]. Therefore, 5-Azax has the ability to enhance the proliferation and differentiation of DPSCs by inhibiting DNA methylation. It was also found that pretreatment of DPSCs with 5-Aza after LPS stimulation reduced the m5C level of the TNF receptor-associated factor 6 (TRAF6) promoter [144]. As a result, inflammation-related

signaling pathways were activated, such as the mitogen-activated protein kinase (MAPK) and nuclear factor-k-gene binding (NF-κB) signaling pathways [144]. Therefore, DNA methylation plays a negative role in the regulation of DPSCs.

In contrast, DNA demethylase has a negative role in inflammatory responses of DPSCs induced by LPS. DPSCs stimulated by LPS increase the expression of TET2. TET2 knockdown downregulates the DNA hydroxymethylation of myeloid differentiation factor 88 (MyD88) promoter and inhibits the NF-κB signaling pathway [145]. Therefore, understanding the regulation of DNA methylation is a potential treatment for pulpitis and periodontitis. Moreover, an in vitro experiment confirmed that the expression of TET1 was significantly increased during the proliferation of PDLSCs [146]. Therefore, the early differentiation and odontogenesis of DPSCs are associated with the level of TET1. Inhibiting the expression of TET1 in DPSCs decreases ALP activity and mineralized nodules during the odontogenic differentiation of DPSCs, which can ultimately inhibit the proliferation and differentiation of DPSCs [147]. During the odontogenic differentiation of DPSCs, deletion of TET1 leads to the downregulation of the family with similarity 20 member C (FAM20C) and enhances the mineralization of DPSCs [148]. FAM20C, also named dentin matrix protein 4 (DMP4), participates in the osteoblastic differentiation of DPSCs [149]. Therefore, TET1 enhances the odontoblastic differentiation of DPSCs by demethylating FAM20C.

4.2.2. Modification of DPSCs by Histone Methylation. Three key markers of histone methylation, H3K4me3, trimethylation on lysine 9 of histone 3 (H3K9me3), and H3K27me3, play a significant role in the regulation of DPSCs [150] (Table 2). For instance, the promoters of early mineralization genes in DPSCs, such as RUNX2, DLX5, and MSX2, all contain H3K4me3 activity markers, which enhance the differentiation of DPSCs [150]. In addition, EZH2 catalyzes the methylation of H3K27me3, while KDM6B demethylates H3K27me3. The expression of EZH2 and H3K27me3 is decreased under inflammatory conditions [151], and the downregulation of EZH2 enhances the differentiation of DPSCs and suppresses the inflammatory response in DPSCs [152]. Moreover, EZH2 inhibition can enhance the osteogenic differentiation of DPSCs by activating β-catenin transcription and the Wnt signaling pathway [153]. Therefore, EZH2 inhibits osteogenic differentiation and promotes the inflammatory response. In contrast, KDM6B (also known as JMJD3), a member of the oxidase family of the JmjC domain, catalyzes the demethylation of H3K27me3 [154]. Under inflammatory conditions, the expression of KDM6B is increased, while H3K27me3 is decreased in DPSCs [151]. KDM6B activates odontogenic transcriptional genes and enhances the odontogenic differentiation of DPSCs by demethylating H3K27me3 of osteogenic genes, such as BMP2 and RUNX2 [154, 155]. Therefore, EZH2 and KDM6B could be applied to potential therapy in regenerating tooth structures. In addition, KDM5A is a histone demethylase (HDM), which is increased during the differentiation and early proliferation of DPSCs [156]. Knockdown of KDM5A enhances odontogenesis and mineralization in DPSCs by increasing H3K4me3 on the promoter of odontogenic marker genes [156]. Therefore, demethylation of H3K4me3 by KDM5A suppresses the dentin differentiation of DPSCs. These results indicated that the demethylation of KDM5A could be applied to dentin repair.

4.2.3. Modification of DPSCs by RNA Methylation. There are also some reports about the regulation of m6A RNA methylation on osteogenic differentiation and proliferation of DPSCs (Table 2). m6A RNA methyltransferases (METTL3 and METTL14) and demethylases (FTO and ALKBH5) exist in PDLSCs [157]. The METTL3-mediated methylation of m6A RNA regulates the proliferation, migration, and differentiation of DPSCs [157, 158]. Bioinformatics analysis found that METTL3 was highly expressed in immature DPSCs and could regulate cell senescence and apoptosis [158]. Deletion of METTL3 resulted in activation of the p53 pathway in DPSCs and had a negative effect on the self-renewal of DPSCs. In addition, METTL3 mediates the expression of Polo-like kinase 1 (PLK1), a mitotic regulator that participates in cell cycle control and senescence apoptosis [158]. The results of in vivo experiments demonstrated that conditional knockout of METTL3 impaired the self-renewal, differentiation, and proliferation of DPSCs, thus damaging tooth root development [159]. The stimulation of LPS increases the expression of METTL3, while knockdown of METTL3 inhibits the expression of inflammation-related factors and inflammation-related signaling pathways, such as MAPK and NF-κB signaling pathways [157]. Therefore, downregulating METTL3-catalyzed RNA methylation of m6A may inhibit the LPS-induced inflammatory response in DPSCs.

4.3. DFSCs. DFSCs can differentiate into alveolar bone, periodontal ligament, and root cementum. As the progenitor cells of some periodontal cell lineages [160–164], DFPCs can differentiate into chondrocytes, osteoblasts, adipocytes, and neural-like cells [4].

4.3.1. Modification of DFSCs by DNA Methylation. DNMT1 can inhibit the osteoblastic differentiation of DFSCs by methylation of HOXA2 (Table 3). Overexpression of the lncRNA HOXA transcript antisense RNA myeloid 1 (HOTAIRM1) inhibits proliferation and enhances osteoblastic differentiation of DFSCs [78, 165, 166]. HOTAIRM1 restrains the DNMT1 and DNA methylation of HOXA2 catalyzed by DNMT1, which promotes the osteogenesis of DFSCs. HOTAIRM1 regulates the methylation state of the HOXA2 gene promoter by controlling DNMT1. Modulating the expression of the HOXA2 gene regulates the osteoblastic differentiation of DFSCs [78, 165, 166]. The upregulation of HOTAIRM1 may prevent the overmethylation of DFSCs from damaging periodontal tissue by inhibiting DNMT1.

4.3.2. Modification of DFSCs by Histone Methylation. H3K4me3 is a marker that activates histone methylation, and H3K9me3 and H3K27me3 are markers of histone methylation that inhibit histone methylation. All of these markers can regulate the differentiation of DFSCs (Table 3). The

Table 3: Methylation and demethylation in DFSCs.

Methylated modification	Epigenetic modifiers	Epigenetic marks	Functions
DNA methylation	DNMT1	DNA methyltransferases	Inhibiting osteogenesis [78, 166].
	5-Aza	DNMT inhibitor	Promoting osteogenesis [78, 166].
Histone methylation		H3K4me3, H3K9me3	H3K4me3 marker will switch to the H3K9me3 marker during osteogenic differentiation [167].
	EZH2	H3K27me3	EZH2 inhibits the osteogenesis of DFSCs by reducing H3K27me3 expression of Wnt gene promoter [111, 150, 168].

Table 4: Methylation and demethylation in SCAPs.

Methylated modification	Epigenetic modifiers	Epigenetic marks	Functions
Histone demethylation	KDM2A	H3K36me2, H3K4me2	Inhibiting osteogenic differentiation [172–177].
	KDM3B	H3K9me2	Exerting a positive impact on osteogenic differentiation of SCAPs and regulating the cell cycle to accelerate the proliferation of SCAPs [178].
	KDM1A	H3K4me2/1, H3K9me2/1	KDM1A forms a protein complex with PLOD2 to inhibit the bone-dentin differentiation [179].
	MLL	H3K4me3	Promoting odontogenic differentiation of SCAPs by upregulating Wnt5a [182].
	KDM6B	H3K27me3	Loss of the demethylase KDM6B increases H3K27me while inhibits odontogenic differentiation [182].

promoters of the important factors that regulate osteogenic differentiation of DFSCs and early mineralization contain the active markers of H3K4me3. During osteogenic differentiation of DFSCs, H3K27me3 displays a prominent effect on DSPP and DMP-1, which are the promoters of dentin formation genes [111, 150, 167]. DFSCs can alter the expression of active H3K4me3, while PDLSCs and ABMSCs cannot. The H3K4me3 marker can turn to the H3K9me3 markers during osteogenic differentiation [167]. The expression of EZH2 and H3K27me3 can be decreased during the osteogenesis of DFSCs. EZH2 inhibits the osteogenesis of DFSCs by decreasing the H3K27me3 level of the Wnt gene promoter [168].

4.4. SCAPs. SCAPs were discovered in the apical papilla of human immature deciduous teeth, and they can differentiate into periapical tissue and pulp tissue [7, 169, 170]. SCAPs can exert vital effects on tooth development, especially in the odontoblasts in the root of the tooth [171]. Under suitable conditions in vitro, SCAPs can differentiate into adipocytes, hepatocytes, osteoblasts, neural cells, and odontoblasts [7, 169, 171].

4.4.1. Modification of SCAPs by Histone Methylation. Recently, the histone methylation involved in the regulation of SCAP fate has attracted increasing attention (Table 4). KDM2A plays a negative role in osteogenic differentiation of SCAPs. The epiregulin (EREG) is a target of KDM2A (also known as FBXL11) that enhances osteogenic differentiation of SCAPs [172]. For instance, EREG is upregulated while KDM2A is downregulated during osteogenic differentiation of SCAPs. The KDM2A/BCL6 corepressor (BCOR) complex restrains histone H3 lysine 36/4 dimethylation (H3K36me2/H3K4me2) of the EREG promoter, which represses the transcription of the EREG promoter and inhibits osteogenic differentiation of SCAPs [172]. In addition, in SCAPs from oculofaciocardiodental (OFCD) syndrome patients, the methylation of H3K4 and H3K36 on the promotor of EREG is increased, which contributes to transcriptional activation of silent genes and enhancement of the osteogenic differentiation potential of SCAPs [173, 174]. However, overexpression of the histone demethylase KDM2A in SCAPs decreases EREG, which inhibits the expression of key transcription factors of bone-dentin differentiation, such as OSX and DLX2, and reduces the bone-dentin differentiation potential of SCAPs [172]. The upregulation of OSX and DLX2 promotes osteogenic differentiation of SCAPs by enhancing ALP activity and calcium mineralization [175]. In addition, secreted frizzled related protein 2 (SFRP2) inhibits NF-κB signaling by repressing the Wnt/β-catenin signaling pathway in hypoxic and inflammatory conditions, and the expression of KDM2A in SCAPs is upregulated while the transcription of SFRP2 is inhibited and the methylation of H3K4me3 and H3K36me2 on the SCAP promoter is decreased [176]. Moreover, the study found that deletion of the demethylase KDM2A in SCAPs increased the methylation of H3K4 and H3K36 in the promoter of SFRP2 and inhibited their transcription. SFRP2 activated OSX and enhanced bone-dentin differentiation, while KDM2A exerted the opposite effect [177]. Therefore, KDM2A inhibits the osteoblastic differentiation of SCAPs by downregulating SFRP.

Lysine-specific demethylase 3B (KDM3B), lysine-specific demethylase 1A (KDM1A), mixed-lineage leukemia (MLL),

and KDM6B also play critical roles in regulating the fate of SCAPs. KDM3B exerts a positive effect on osteoblastic differentiation of SCAPs by upregulating the expression of OCN, RUNX2, OSX, and DSPP. Moreover, KDM3B is involved in regulating the cell cycle to accelerate the proliferation of SCAPs [178]. In vitro, knockdown of KDM1A in SCAPs downregulates ALP activity and calcium mineralization of SCAPs. KDM1A combines with PLOD2 to form a protein complex that inhibits the bone-dentin differentiation of SCAPs [179]. MLL is a methylase of H3K4me3, KDM6B is the demethylase of H3K27me, and both promote odontogenic differentiation of SCAPs by upregulating Wnt5a [180–182]. The transcriptional activity of Wnt5a is regulated by H3K27me3 and H3K4me3. In addition, deficiency of the demethylase KDM6B enhances H3K27me3 and inhibits odontogenic differentiation [182].

5. Conclusion

Growing attention has been given to the differentiation, self-renewal, and regulation of DMSCs in the field of regenerative medicine [183–185]. For instance, DPSCs are used for the treatment of mandible defects and periodontal regeneration in chronic periodontitis patients [48, 186–188]. An ABMSC-based treatment strategy in the periodontal reconstruction of 27 patients enhanced periodontal tissue healing, periodontal bone reconstruction, reduction of probing pocket depth (mean ± SD, 0.75 ± 5.5 mm), and no adverse reactions after 12 months [189]. PDLSCs were filled into 10 patients with periodontal bone defects, and periodontal probing depth was reduced (3.2 ± 1.9 mm) while radiographic bone height was increased (2.3 ± 1.8 mm) in all 10 cases without serious adverse reactions in a clinical trial after 6 months [190]. The application of DMSCs is an opportunity for regenerative medicine, including the treatment of dental caries, pulp necrosis, periapical disease, and so on. However, the mechanism of the biology and regenerative ability of DMSCs requires further study.

Methylation modifications tightly and precisely regulate the fate of SC differentiation. For example, DNMT-mediated DNA methylation is essential for ESC differentiation [191]. Because of the lack of maintenance of DNMT1, extensive nonCpG methylation at CpA dinucleotides exists in ESCs [192]. Moreover, METTL3-/- mice showed abnormal differentiation of ESCs in vitro. METTL3 is a writer of m6A RNA methylation that can enhance the differentiation and reduce the self-renewal of ESCs [193, 194]. Furthermore, methylation modifications of SCs have been applied to clinical therapies. 5-AZA, as a DNMT inhibitor targeting brain cancer SCs, has been used in phase I trials to treat brain cancer [53]. It is also used to treat juvenile mononuclear leukemia (JMML) in conjunction with allogeneic hematopoietic stem cell transplantation (HSCT).

Recently, studies related to the cell proliferation and immune regulation of DMSCs by methylation modifications have attracted more attention [157, 158]. For example, the methyltransferase SETD1B catalyzed H3K4 histone trimethylation when DMSCs were stimulated by LPS, which increased H3K4me3 of gene promoters on IL-1β and IL-6, thus activating inflammatory signaling pathways and releasing inflammatory cytokines [125]. Knockdown of METTL3 and TET2 downregulated MyD88 and inhibited LPS-induced inflammatory responses in DPSCs [145, 157]. Therefore, understanding the regulation of methylation modifications on the immunoinflammatory responses involved in DMSCs in periodontitis contributes to further study of the mechanism of methylation on inflammation.

Increased DNA methylation and histone methylation have been reported to regulate the osteogenic differentiation of DMSCs, including PDLSCs, DPSCs, DFSCs, and SCAPs. More attention should be given to the regulation of RNA methylation in DMSCs. Additionally, further studies are also needed to study the methylation modification of DMSC proliferation, autophagy, apoptosis, and migration. Current studies still focus on animal experiments. For instance, DLX3 knockout in Wnt1-cre neural crest deletion mice causes major dentin defects [195]. METTL3 conditional knockout mice present molar root dysplasia [159]. Glucose inhibited the activation of DNMT1 and TNFR-1 [118], while TNF-α receptor p55-deficient mice showed less alveolar bone loss in periodontal tissues [196]. Kdm3C KO mice were more sensitive to LPS and showed increased alveolar bone loss [197]. Osr2-Cre; Ezh2fl/fl mice exhibited EZH2 participation in root patterning during molar root development [198]. In the future, more studies focusing on the methylation modifications of DMSCs, especially applications in tissue engineering and regulation of inflammation, are needed. These studies provide the basis for the therapy of oral disease and tissue regeneration applications.

Abbreviations

DMSCs:	Dental mesenchymal stem cells
DPSCs:	Dental pulp stem cells
DFSCs:	Dental follicle stem cells
PDLSCs:	Periodontal ligament stem cells
SCAPs:	Stem cells from apical papillae
SHED:	Stem cells from exfoliated deciduous teeth
ABMSCs:	Stem cells from alveolar bone
GMSCs:	Stem cells from gingival tissue
KDM6B:	Lysine (K)-specific demethylase 6B
KDM2A:	Lysine demethylase 2A
H3K27me2/3:	Demethylation/trimethylation on lysine 27 of histone 3
BMP2:	Bone morphogenetic protein 2
5-Aza:	5-Aza-2′-deoxycytidine
SCs:	Stem cells
TGSCs:	MSCs derived from the tooth germ.
SAM:	S-Adenosyl methionine
SAH:	S-Adenosyl homocysteine
DNMTs:	DNA methyltransferases
HSCs:	Hematopoietic stem cells
TET:	Ten–eleven translocation
TET1:	Ten–eleven translocation 1
ESCs:	Embryonic stem cells
TET2:	Ten–eleven translocation 2
5-mC:	5-Methylcytosine

5-hmC: 5-Hydroxy methylcytosine
TDG: Thymine DNA glycosylase
PRMTs: Protein arginine methyltransferases
PKMTs: Protein lysine methyltransferases
DOT1L: Telomeric silencing 1 like
JmjC: Jumonji C-terminal
LSD1: Lysine-specific demethylase 1
IGFBP5: Insulin-like growth factor binding protein 5
H3K4: Histone H3 at lysine 4
m1A: N1-Methyladenosine
m6A: N6-Adenine methylation
m7G: 7-Methyl guanosine
METTL3/14: Methyltransferase-like protein 3/14
FTO: Obesity-associated protein
ALKBH5: Fat mass alkB homolog 5
GSCs: Glioblastoma stem-like cells
RUNX2: Runt-related transcription factor 2
ALP: Alkaline phosphatase
OPN: Osteopontin
DFATs: Dedifferentiated fat cells
LPS: Lipopolysaccharide
HG: High glucose
EZH2: Enhancer of zeste homolog 2
OSX: Osterix
E2F: Transcription factor 2
TNF-α: Tumor necrosis factor α
SETD1B: SET domain-containing 1B
TNFR-1: Tumor necrosis factor-alpha receptor-1
DKK-1: Dickkopf-related protein-1
hnRNPL: Heterogeneous nuclear ribonucleoprotein L
KLF2: Kruppel-like factor 2
SETD2: SET domain-containing protein 2
KDM6A: Lysine- specific demethylase6A
H3K4me3: Trimethylation on lysine 4 of histone 3
GPNMB: Glycoprotein nonmetastatic melanoma protein B
PRDM9: PR domain containing 9
miR-675: MicroRNA-675
DLX3: Distal-less homeobox 3
KLF4: Kruppel-like factor 4
DSPP: Dentin sialophosphoprotein
DMP-1: Dentin matrix protein-1
NF-κB: Nuclear factor-κB
TRAF6: TNF receptor-associated factor 6
MAPK: Mitogen-activated protein kinase
MyD88: Myeloid differentiation factor 88
FAM20C: Family with sequence similarity 20, member
H3K9me3: Trimethylation on lysine 9 of histone 3
DMP4: Dentin matrix protein 4
HDMs: Histone demethylases
EREG: Epithelial regulatory factor
HOTAIRM1: lncRNA HOXA transcript antisense RNA myeloid 1
PLK1: Polo-like kinase 1
H3K36me2/H3K4me2: Histone H3 lysine 36/4 dimethylation
BCOR: BCL6 corepressor
OFCD: Oculofaciocardiodental
SFRP2: Secret frizzled-related protein 2
KDM3B: Lysine-specific demethylase 3B
KDM1A: Lysine-specific demethylase 1A
MLL: Mixed-lineage leukemia
JMML: Juvenile mononuclear leukemia
HSCT: Hematopoietic stem cell transplantation.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

All authors have made substantial, direct, and intellectual contributions to the work. At the same time, all authors participated in designing the study, drafting and writing the manuscript, and approving it for submission. Hui Zhang and Hong Fu contributed equally to this work.

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Research Article

Glycogen Synthase Kinase 3β inhibits BMSCs Chondrogenesis in Inflammation via the Cross-Reaction between NF-κB and β-Catenin in the Nucleus

Zhenggang Wang,[1] Zhiyi He,[1] Weikai Zhang,[1] Shuang Liang,[1] Kun Chen,[2] Shimeng Xu,[1] Ying Zhang,[3] and Peng Cheng[1]

[1]*Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*
[2]*Department of Orthopedics, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, China*
[3]*Department of Nephrology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

Correspondence should be addressed to Peng Cheng; chengpeng2015@tjh.tjmu.edu.cn

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Inflammation can influence the pluripotency and self-renewal of mesenchymal stem cells (MSCs), thereby altering their cartilage regeneration ability. Sprague-Dawley (SD) rat bone marrow mesenchymal stem cells (BMSCs) were isolated and found to be defective in differentiation potential in the interleukin-1β- (IL-1β-) induced inflammatory microenvironment. Glycogen synthase kinase-3β (GSK-3β) is an evolutionarily conserved serine/threonine kinase that plays a role in numerous cellular processes. The role of GSK-3β in inflammation may be related to the nuclear factor-κB (NF-κB) signaling pathway and the Wnt/β-catenin signaling pathway, whose mechanism remains unclear. In this study, we found that GSK-3β can inhibit chondrogenesis of IL-1β-impaired BMSCs by disrupting metabolic balance and promoting cell apoptosis. By using the inhibitors LiCl and SN50, we demonstrated that GSK-3β regulates the chondrogenesis via the NF-κB and Wnt/β-catenin signaling pathways and possibly mediates the cross-reaction between NF-κB and β-catenin in the nucleus. Given the molecular mechanisms of GSK-3β in chondrogenic differentiation in inflammation, GSK-3β is a crucial target for the treatment of inflammation-induced cartilage disease.

1. Introduction

Articular cartilage damage is a frequent clinical problem with joint swelling and pain [1]. As the main character of degenerative joint diseases, mainly osteoarthritis and chronic inflammatory joint disease, such as rheumatoid arthritis, it is important to study the mechanism of cartilage damage and how to promote cartilage repair [2, 3]. Bone marrow mesenchymal stem cells (BMSCs) can be induced to differentiate into chondrocytes under certain conditions, therefore they have a potential application in the treatment of cartilage damage, inflammation, and other cartilage diseases [4, 5].

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine-protein kinase involved in many intracellular functions [6]. And it is important in regulating the activity of nuclear factor-κB (NF-κB) [7, 8]. The NF-κB signaling pathway plays an essential role in inflammation by regulating the transcription of genes involved in cell growth and cell death. It is reported that NF-κB influences chondrogenic differentiation by down-regulating the mRNA levels of Sox9, a chondrogenic transcription factor [9]. Meanwhile, the expression

Table 1: The information of primers.

Gene		Sequences
Sox9	Forward	5′-GTGGGAGCGACAACTTTACC-3′
	Reverse	5′-GCGAGCACTTAGCAGAGGC-3′
Collagen 2a	Forward	5′-CACCCAGAGTGGAAGAGCG-3′
	Reverse	5′-TCAGTGGACAGTAGACGGAGGA-3′
Aggrecan	Forward	5′-CAAACAGCAGAAACAGCCAAGT-3′
	Reverse	5′-GAAGGCATAAGCATGTGAAAGTG-3′
GAPDH	Forward	5′-AACGACCCCTTCATTGACCTC-3′
	Reverse	5′-CCTTGACTGTGCCGTTGAACT-3′

of NF-κB p65 in growth plate chondrocytes has been shown to facilitate growth plate chondrogenesis [10].

In addition, GSK-3β earmarks β-catenin for degradation by the proteasome, which mediates the Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway widely participates in cellular differentiation, especially in chondrogenesis and osteogenesis [11, 12]. And aberrations in the Wnt/β-catenin signaling pathway are often associated with defective cellular differentiation. However, the role of GSK-3β in the BMSCs chondrogenesis in IL-1β-induced inflammation has not been fully described, and the underling mechanisms deserve exploring.

In this study, we investigated the effect of GSK-3β on IL-1β-impaired chondrogenesis of BMSCs. Meanwhile, we used LiCl, an inhibitor of GSK-3β, to explore the regulation of GSK-3β on the NF-κB and Wnt/β-catenin signaling pathways. Furthermore, SN50, a specific inhibitor of NF-κB translocation, was used to verify the cross-reaction between NF-κB and β-catenin in the nucleus.

2. Materials and Methods

2.1. Cell Culture and Chondrogenic Differentiation. Sprague-Dawley (SD) male rat BMSCs were purchased from Cyagen Biosciences (RASMX-01001) and were cultured with DMEM/F12 medium (BOSTER, PYG0084) containing 10% fetal bovine serum (Gibico, 10100147) at 37°C with 5% CO_2 in a humidified incubator. BMSCs were passaged according to the usual method when they were 90% confluent, and the third passage was used in the experiments. Approximately 2.5×10^5 BMSCs were transferred into a 15 mL reaction tube and centrifuged at 150 g for 5 min at room temperature. After incubation in 0.5 mL chondrogenic differentiation medium (CDM; Cyagen Biosciences, RAXMX-90041) at 37°C with 5% CO_2 for 24 h, the pellets had a round morphology and floated within the medium. Then based on previous researches, the pellets were stimulated with 10 ng/mL IL-1β (PeproTech, 211-11B), 10 nM GSK-3β (Creative BioMart, 2728R), 10 mM LiCl (Sigma-Aldrich, L9650), and/or 15 μM SN50 (MedChemExpress, HY-P0151) [13–16]. And the CDM was carefully replaced every 2-3 days. After 3 weeks, the pellets were collected and chondrogenic differentiation was evaluated. In addition, BMSCs were seeds at 1×10^5 cells per well in 6-well plates. After incubation for 24 h, the medium was change to the CDM, and BMSCs were stimulated at the same regimen described above. On day 3, BMSCs were harvested to detect apoptosis and explore the mechanisms.

2.2. Histological Analysis of Chondrogenic Pellet. The pellets were washed with PBS, fixed with 4% paraformaldehyde for three hours and prepared for paraffin embedding. Then pellets were fixed to a paraffin block and 3 μm sections were obtained using a microtome. For Alcian blue staining, sections were incubated in 1% Alcian blue solution for 30 minutes. For immunofluorescent staining, sections were incubated with 10% donkey serum for 30 min after dewaxing and antigen retrieval. Then sections were incubated with anti-Col 2a antibody (Santa Cruz Biotechnology, sc-52658, 1 : 50) overnight at 4°C. The next day, sections were incubated with FITC conjugated secondary antibody at 37°C for 1 h in the dark. Finally, sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Solarbio, C0065) for 5 min and then visualized under a fluorescence microscope (Nikon, Japan).

2.3. DMMB Assay of GAG. At the end of the experiment, the conditioned medium was collected, and glycosaminoglycan (GAG) was quantified using a dimethyl-methylene blue (DMMB; Sigma-Aldrich, USA) assay. Briefly, for each well of 96-well plate, 40 μL sample or standard was mixed with 250 μL DMMB solution and incubated at 37°C for 1 h. 525 nm absorption was measured on a microplate ELISA reader. The amount of GAG was calculated by the OD value according to the chondroitin sulfate (Sigma-Aldrich, USA) standard curve.

2.4. RNA Extraction and qRT-PCR Analysis. Total RNA was extracted from the pellets using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription was performed using a Superscript III first-strand cDNA synthesis kit (Thermo Fisher Scientific, USA). The expression levels of chondral-related genes Sox9, Collagen 2a, and Aggrecan were assessed by quantitative real-time PCR with SYBR-Green master mix (Thermo Fisher Scientific, USA). GAPDH was selected as an internal

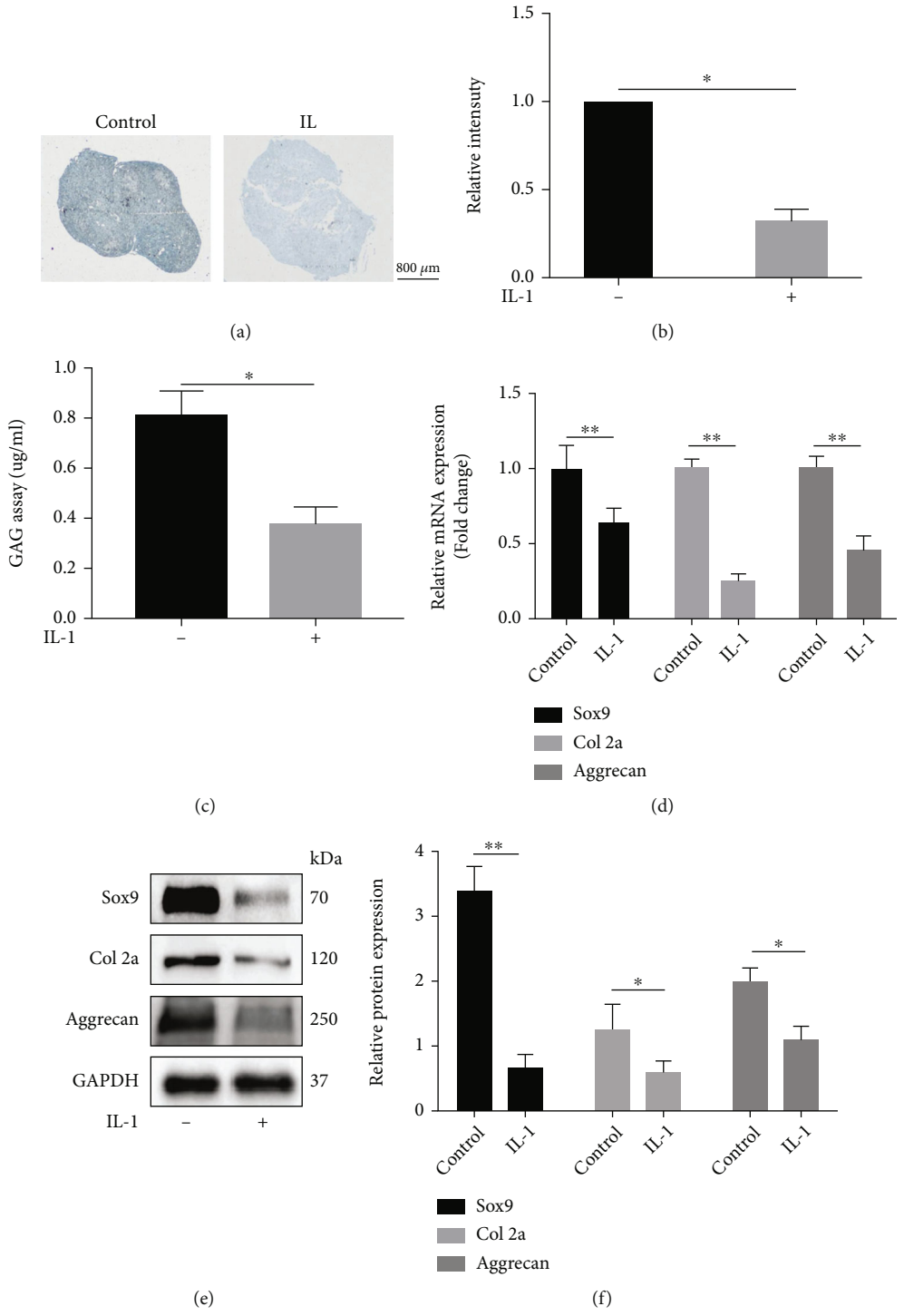


Figure 1: Chondrogenic differentiation of BMSCs in an IL-1β-induced inflammatory microenvironment. (a) and (b) Representative images and intensity quantification of Alcian blue staining. (c) The GAG content in the culture medium. The expression level of Sox9, Collagen 2a and Aggrecan were detected by qRT-PCR (d) and western blotting (e) and (f). All results are expressed as the mean ± SD ($n = 3$). NS: not significant, $^{*}P < 0.05$ and $^{**}P < 0.01$. Bar = 800 μm.

control. Gene expression levels were normalized to GAPDH by using the $\Delta\Delta$Ct method. The primers are listed in Table 1.

2.5. Western Blotting Analysis. Total proteins were collected from the pellets or BMSCs. And the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, P0027) was used to extract proteins from BMSCs. Proteins were separated in 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Then membranes were blocked with 5% BSA for 1 h at room temperature and incubated with primary antibody overnight at 4°C. Specific bands were detected with an HRP-conjugated secondary antibody and

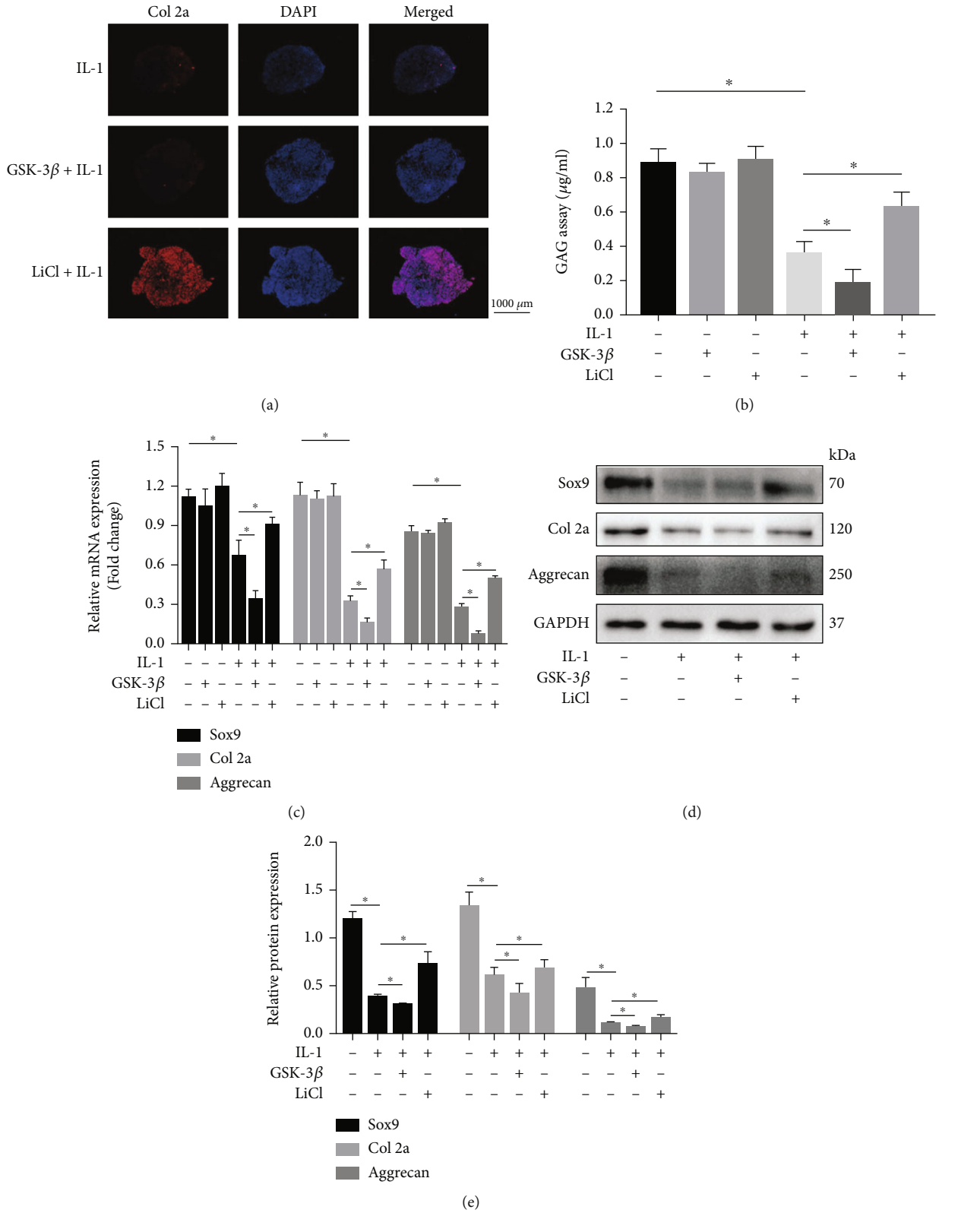


FIGURE 2: GSK-3β disrupts metabolic balance of IL-1β-impaired BMSCs. (a) Representative immunofluorescence images of collagen 2a. (b) The GAG content in the culture medium. (c) The expression level of Sox9, C and Aggrecan were detected by qRT-PCR (c) and western blotting (d) and (e). All results are expressed as the mean ± SD ($n = 3$). NS: not significant and $^*P < 0.05$. Bar = 1000 μm.

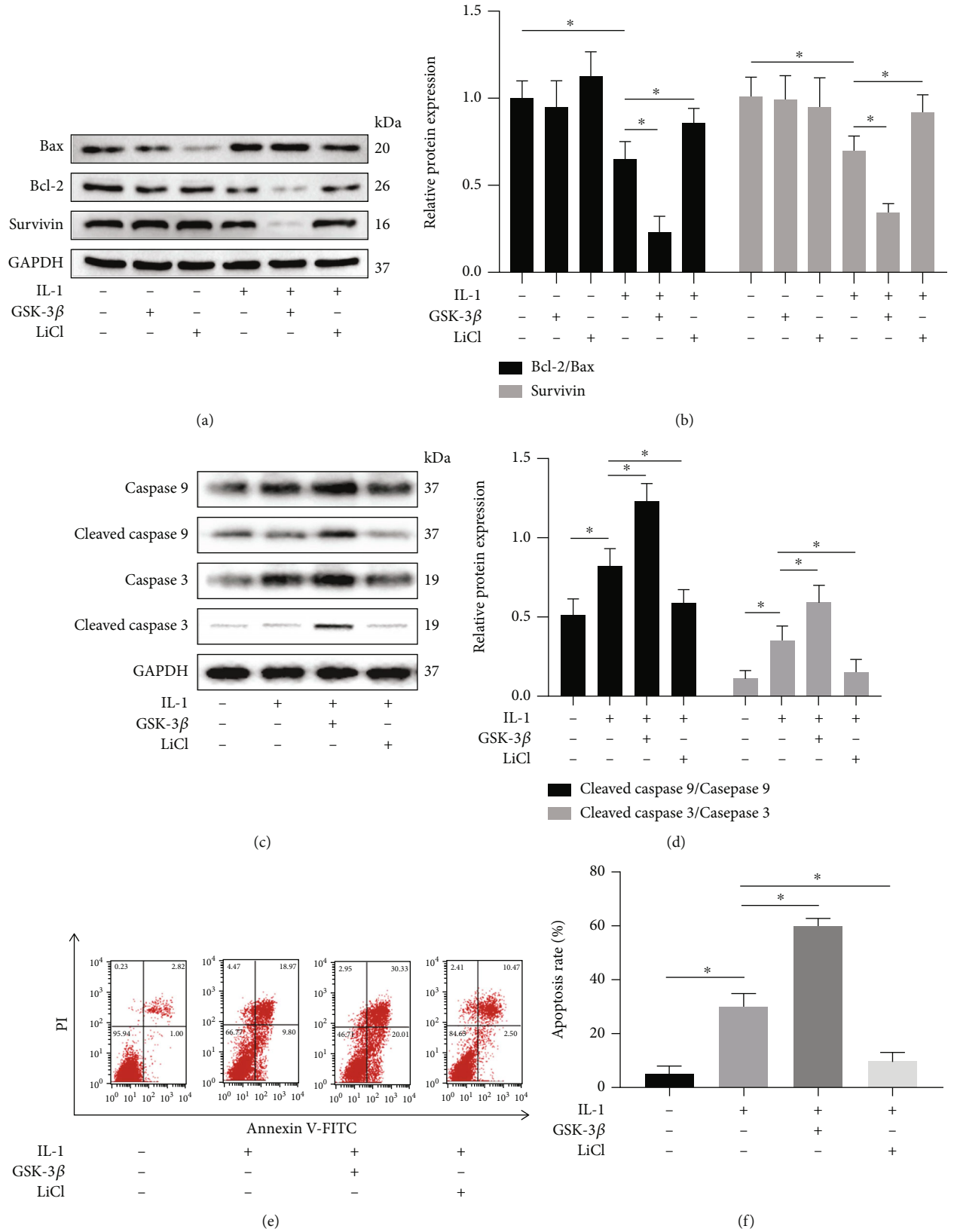
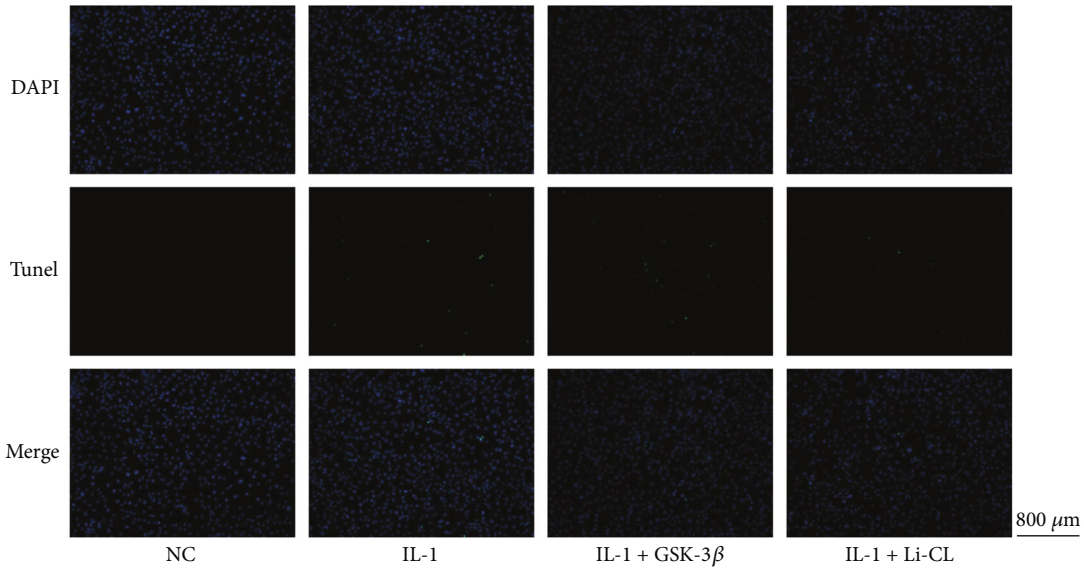


Figure 3: Continued.



(g)

Figure 3: GSK-3β promotes apoptosis of IL-1β-impaired BMSCs. (a)-(d) The expression level of caspase 9, cleaved caspase 9, caspase 3, cleaved caspase 3, Bax, Bcl-2, and Survivin were detected by western blotting. Flow cytometric analysis (e) and (f) and TUNEL staining (g) were performed to assess the number of apoptotic cells. All results are expressed as the mean ± SD ($n = 3$). NS: not significant, $^*P < 0.05$. Bar = 800 μm.

were visualized by an enhanced chemiluminescence kit. The expression of protein was normalized to GAPDH (Biotechnology, 60004-1-Ig) or Histone (Abcam, ab179) using Image Lab software (Bio-Rad, USA). For total proteins from the pellets, primary antibodies against Sox9 (CST, 82630), Col 2a, and Aggrecan (Biotechnology, 13880-1-AP) were used. For total proteins from BMSCs, primary antibodies against Bax (CST, 14796), Bcl-2 (Abcam, ab196495), Survivin (CST, 2808), caspase 9 (CST, 9508), cleaved caspase 9 (CST, 9507), caspase 3 (CST, 9662), cleaved caspase 3 (CST, 9661), IKKβ (CST, 8943), p-IKKα/β-Ser176/Ser180 (CST, 2697), IκBα (CST, 9242), p-IκBα-Ser32 (CST, 2859), NF-κB p65 (CST, 8242), p-NF-κB p65 (CST, 3033), β-catenin (CST, 8480), p-β-catenin (CST, 4176), GSK-3β (CST, 12456), and p-GSK-3β (CST, 5558) were used. For nuclear and cytoplasmic protein from BMSCs, primary antibodies against NF-κB p65 and β-catenin were used.

2.6. Apoptosis Evaluation. The apoptotic rate of BMSCs was determined using the flow cytometry (FCM) analysis with an Annexin V-FITC/PI Apoptosis kit (BD Bioscience, USA). BMSCs were harvested and resuspended in a 500 μL binding buffer and stained with 5 μL FITC Annexin V and 5 μL propidium iodide (PI). After incubation for 15 min, the samples were tested using a flow cytometer (BD Bioscience, USA). For each FCM analysis, 10,000 events were recorded. Annexin V+/PI- and Annexin V+/PI+ cells were considered as early and late phase apoptotic cells.

TUNEL staining was performed on BMSCs cultured on 12-well plates using a one-step TUNEL assay Kit (Beyotime, China). According to the manufacturer's instructions, the samples were washed with PBS and fixed with 4% paraformaldehyde for 30 min. Then the samples were incubated with 50 μL of TUNEL detection liquid for 60 min at 37°C in the dark. Finally, nuclei were stained with DAPI and samples were visualized under a fluorescence microscope.

2.7. Immunofluorescence. BMSCs were harvested and resuspended in PBS. The suspension was smeared to glass slide and was dried. Cells were fixed with 4% paraformaldehyde, permeabilized in 0.1% TritonX-100, and blocked with 3% BSA. Thereafter, cells were incubated with the primary antibody in blocking buffer against NF-κB p65 and β-catenin at 4°C overnight. The next day, cells were incubated with FITC conjugated and TRITC conjugated secondary antibody at 37°C for 1 h in the dark. Finally, cells were stained with DAPI and visualized under a laser confocal microscope (Olympus, Japan).

2.8. Statistical Analysis. All experimental group were independently performed in biological triplicate. Data were presented as means ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, USA). Student's t-test was used to assess differences between the two groups. One-way ANOVA was used to assess differences among multiple groups. P values <0.05 were considered statistically significant.

3. Results

3.1. Chondrogenic Differentiation of BMSCs in an IL-1β-Induced Inflammatory Microenvironment. BMSCs were formed into pellets and cultured in the CDM for 21 days. To establish an inflammatory microenvironment, IL-1β was added into the CDM from day 1. The Alcian blue staining intensity of the IL-1β group was much lower than those of the control group (Figures 1(a) and 1(b)). And GAG

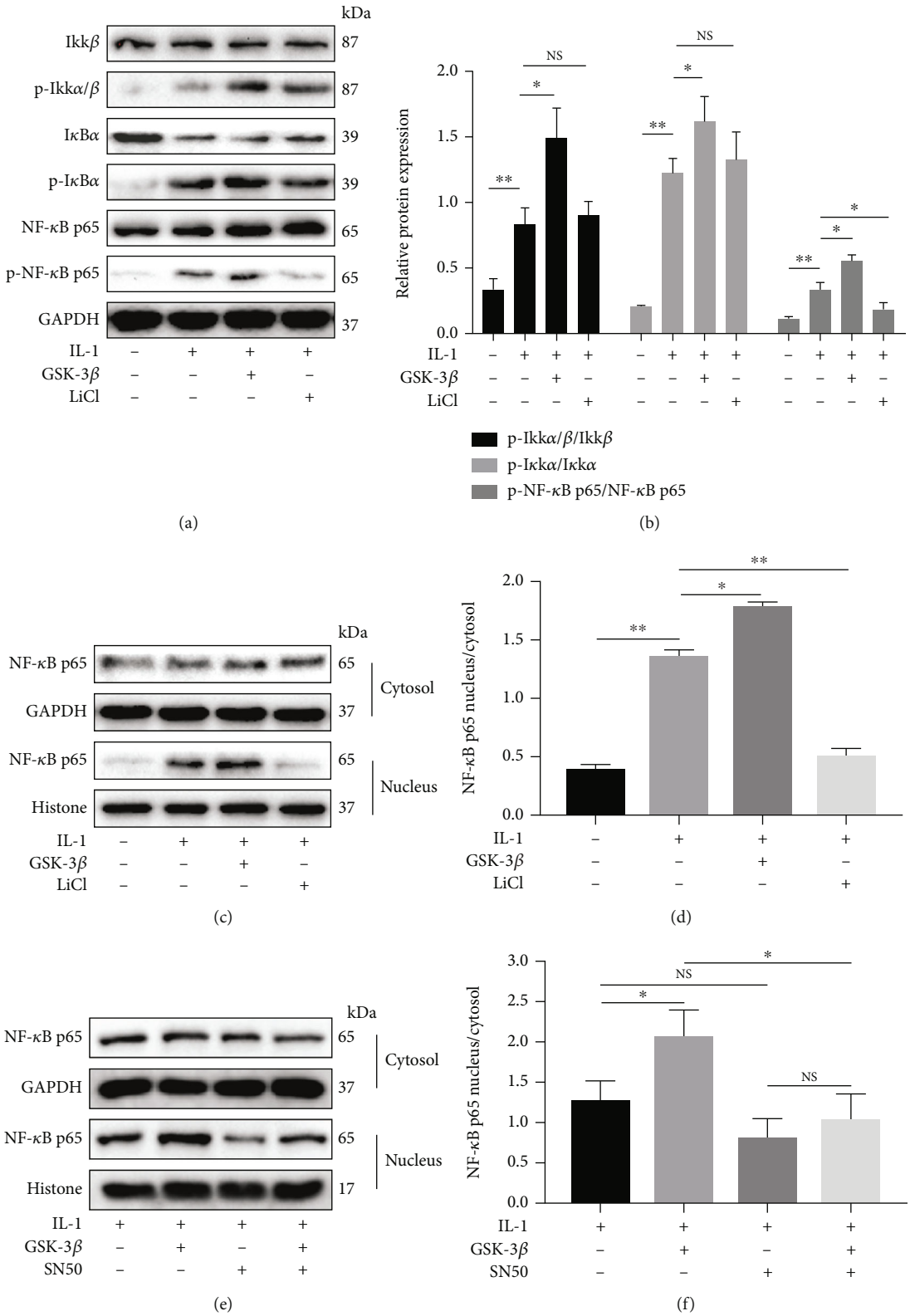


Figure 4: GSK-3β regulates the NF-κB signaling pathway in IL-1β-induced inflammation. Phosphorylation and/or total expression of IKKβ, IKKα/β, IκBα, and NF-κB p65 (a) and (b) were detected by western blotting. Visualization and quantification of the expression of cytoplasmic (c) and (d) and nuclear (e) and (f) NF-κB p65. All results are expressed as the mean ± SD ($n = 3$). NS: not significant, $^{*}P < 0.05$, and $^{**}P < 0.01$.

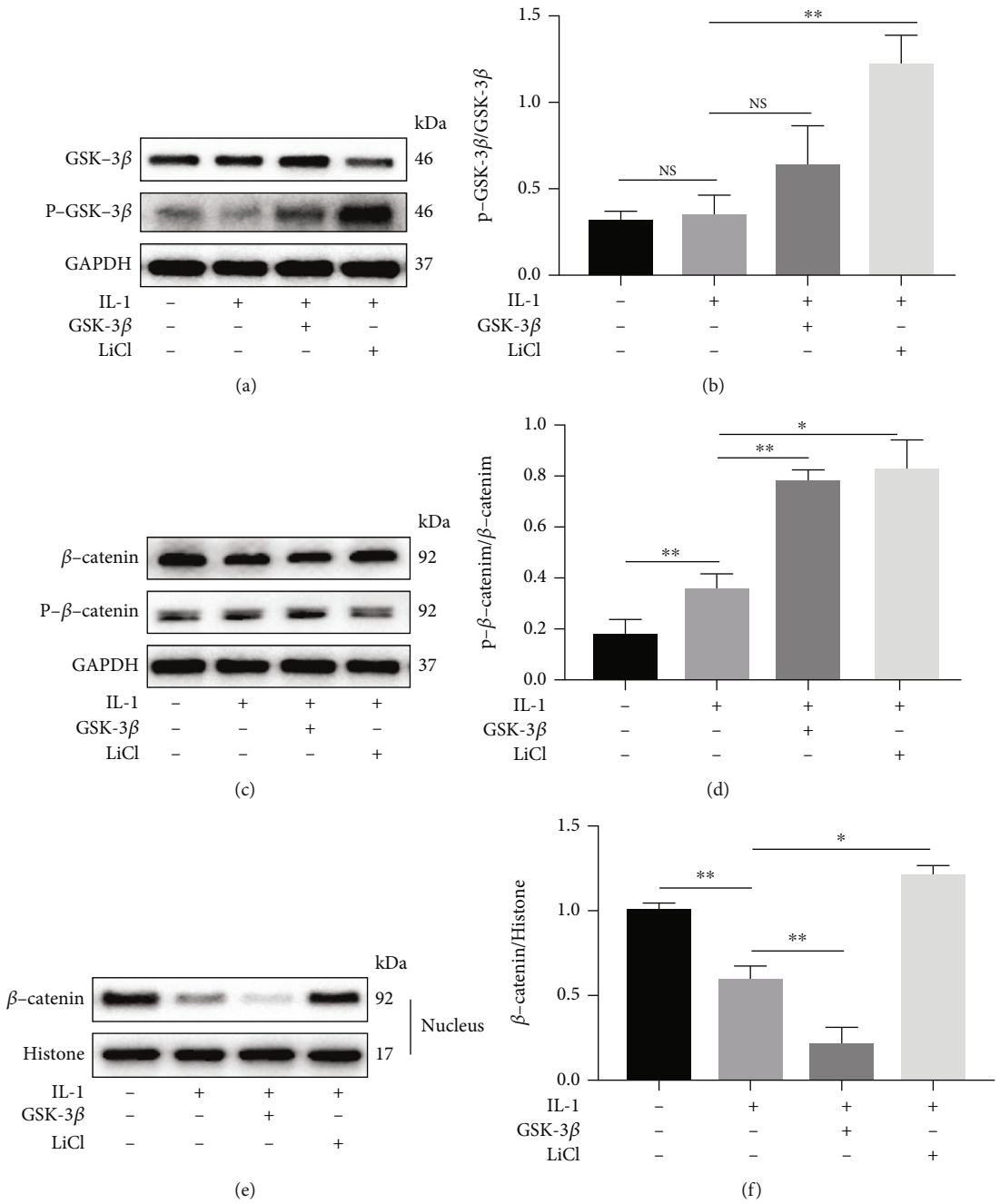


Figure 5: Continued.

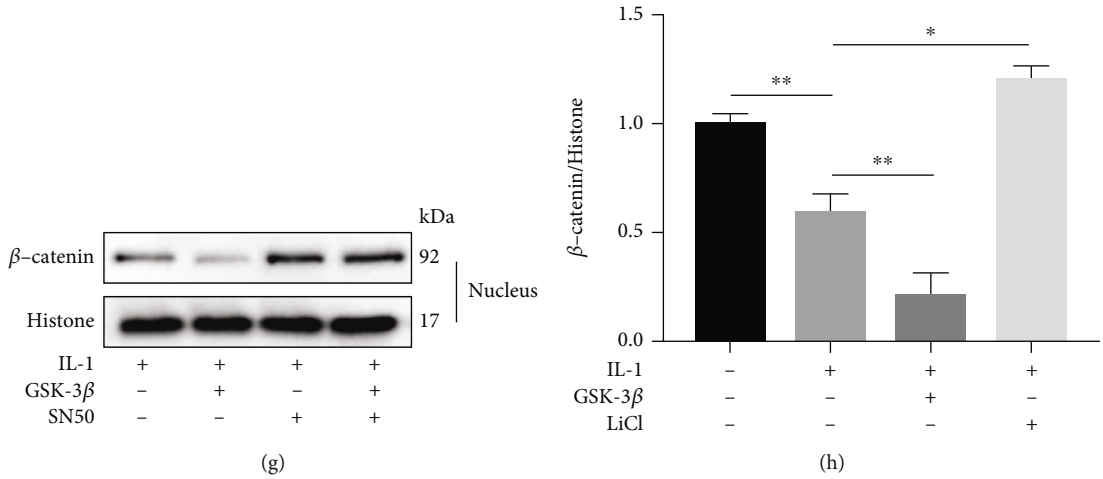


Figure 5: GSK-3β regulates the Wnt/β-catenin signaling pathway in IL-1β-induced inflammation. Phosphorylation and total expression of GSK-3β (a) and (b) and β-catenin (c) and (d) were detected by western blotting. (e)-(h) visualization and quantification of the expression of nuclear β-catenin. All results are expressed as the mean ± SD ($n = 3$). NS: not significant, $^{*}P < 0.05$, and $^{**}P < 0.01$.

quantity in the conditioned medium also decreased in the IL-1β group (Figure 1(c)). Besides, IL-1β significantly decreased the expression of Sox9, Collagen 2a (Col 2a), and Aggrecan at both gene and protein levels (Figures 1(d)–1(f)).

3.2. GSK-3β Disrupts Metabolic Balance of IL-1β-Impaired BMSCs. Immunofluorescence staining showed that compared with the IL-1β group, the expression of Collagen 2a was decreased in the GSK-3β+IL-1β group, while was significantly increased in the LiCl+IL-1β group (Figure 2(a)). Compared with the control group, GAG quantity in the conditional medium was not changed by GSK-3β or LiCl alone, and GSK-3β enhanced and IL-1β-induced decline in GAG quantity, while LiCl reversed this trend (Figure 2(b)). Accordingly, the expression of Sox9, Col 2a, and Aggrecan were significantly decreased in the GSK-3β+IL-1β group than in the IL-1β group at both gene and protein levels (Figures 2(c)–2(e)).

3.3. GSK-3β Promotes Apoptosis of IL-1β-Impaired BMSCs. Western blotting analysis was used to detect the expression of Bax, Bcl-2, and Survivin (Figures 3(a) and 3(b)). We found that GSK-3β or LiCl only had no significant effect on the expression of these proteins (although LiCl reduced Bax expression, there was no statistical significance), and IL-1β increased Bax expression and inhibited Bcl-2 and Survivin expression. At the same time, the cointervention with GSK-3β enhanced these trends, while LiCl reversed them. Meanwhile, the expression of caspase 9, cleaved caspase 9, caspase 3, and cleaved caspase 3 were also detected, and the results were consistent (Figures 3(c) and 3(d)). In addition, the flow cytometric analysis (Figures 3(e) and 3(f)) and TUNEL staining (Figure 3(g)) were performed to assess the number of apoptotic cells. IL-1β markedly increased the cell apoptosis compared to the control group, and the addition of GSK-3β exacerbated this effect, while LiCl reversed it.

3.4. GSK-3β Regulates the NF-κB and Wnt/β-Catenin Signaling Pathways in IL-1β-Induced Inflammation. To explore the possible molecular mechanism, we investigated the NF-κB and Wnt/β-catenin signaling pathways in BMSCs. In this study, IL-1β significantly stimulated the phosphorylation of IKKα/β, IκBα, and NF-κB p65, which was enhanced by the addition of GSK-3β. Interestingly, only NF-κB phosphorylation was reversed by LiCl (Figures 4(a) and 4(b)). In addition, we found that the phosphorylation of GSK-3β was significantly increased in the LiCl+IL-1β group. And compared with the IL-1 group, the phosphorylation of β-catenin was increased in the GSK-3β+IL-1β group while decreased in the LiCl+IL-1β group (Figures 5(a)–5(d)).

3.5. GSK-3β Mediates the Cross-Reaction between NF-κB and β-Catenin in the Nucleus. Furthermore, the expression of NF-κB and β-catenin in the cytosol and nucleus were detected. IL-1β increased NF-κB expression in the nucleus, and GSK-3β enhanced this trend, while LiCl and SN50 reversed it (Figures 4(c)–4(f)). Meanwhile, IL-1β decreased β-catenin expression in the nucleus, and GSK-3β enhanced this trend, while LiCl reversed it (Figures 5(e) and 5(f)). Newly, after cointervention with SN50, a specific inhibitor of NF-κB translocation, β-catenin expression in the nucleus was significantly increased (Figures 5(g) and 5(h)). Meanwhile, immunofluorescence was performed (Figure 6). The results showed that GSK-3β promoted the IL-1β-induced nuclear translocation of NF-κB p65, while LiCl and SN50 decreased it. And in the IL-1β group and the GSK-3β+IL-1β group, the green fluorescence signal (β-catenin) could be clearly observed in the cytoplasm, but there was almost no signal in the nucleus. However, in the LiCl+IL-1β group, the SN50+IL-1β group and the SN50+GSK-3β+IL-1β group, the green fluorescence signal in cytoplasm was significantly reduced, and only slight signal was observed in the nucleus.

4. Discussions

Bone marrow mesenchymal stem cells can be induced to differentiate into chondrocytes under certain conditions, which has potential application value in the treatment of cartilage diseases [17]. In agreement with previous studies, we showed

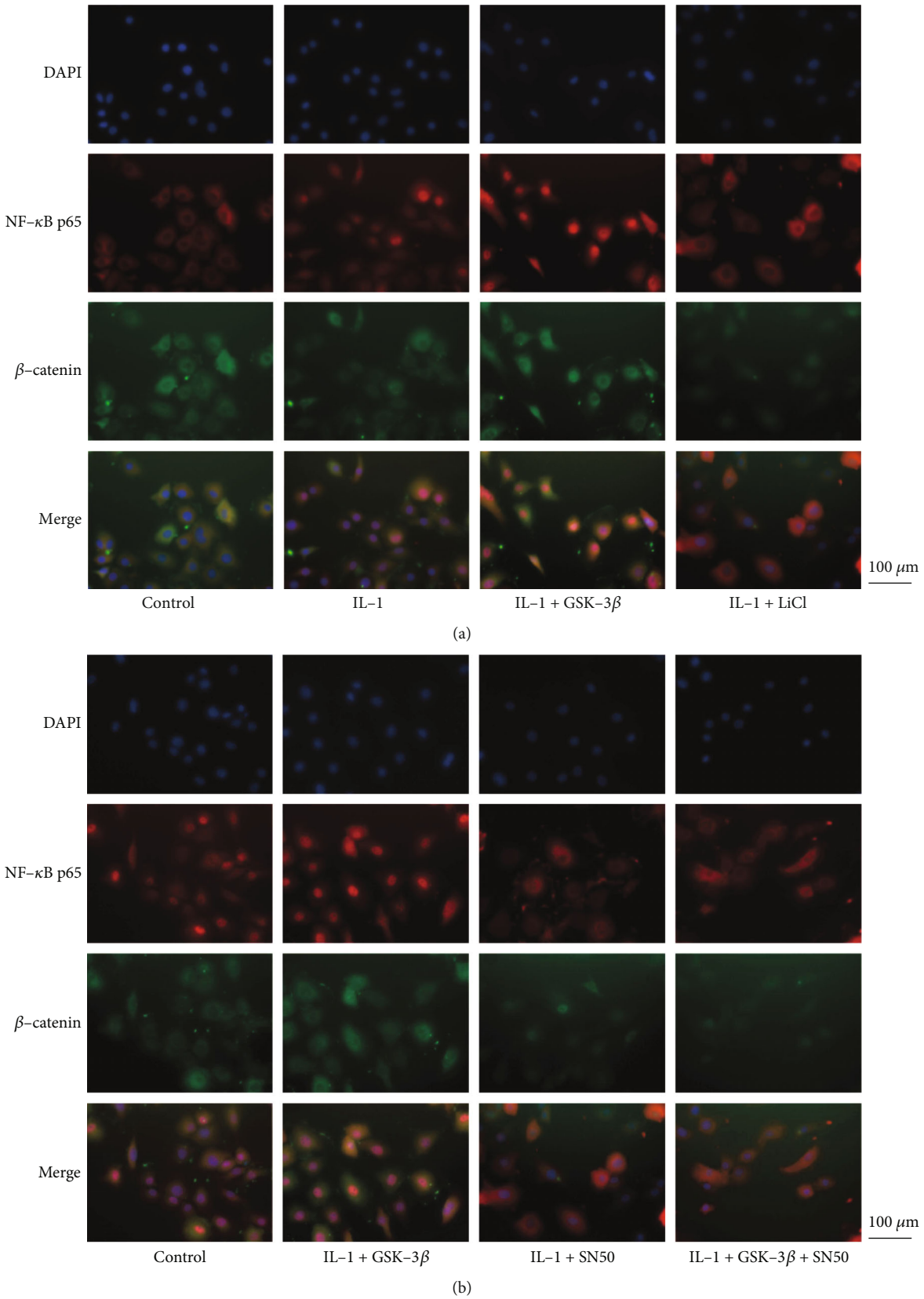


Figure 6: GSK-3β mediates the cross-reaction between NF-κB and β-catenin in the nucleus. Immunofluorescence was used to observe the distribution of NF-κB p65 (red) and β-catenin (green). The nuclei were DAPI-stained (blue). Three independently repeated experiments were performed, and a representative image is shown. Bar = 100 μm.

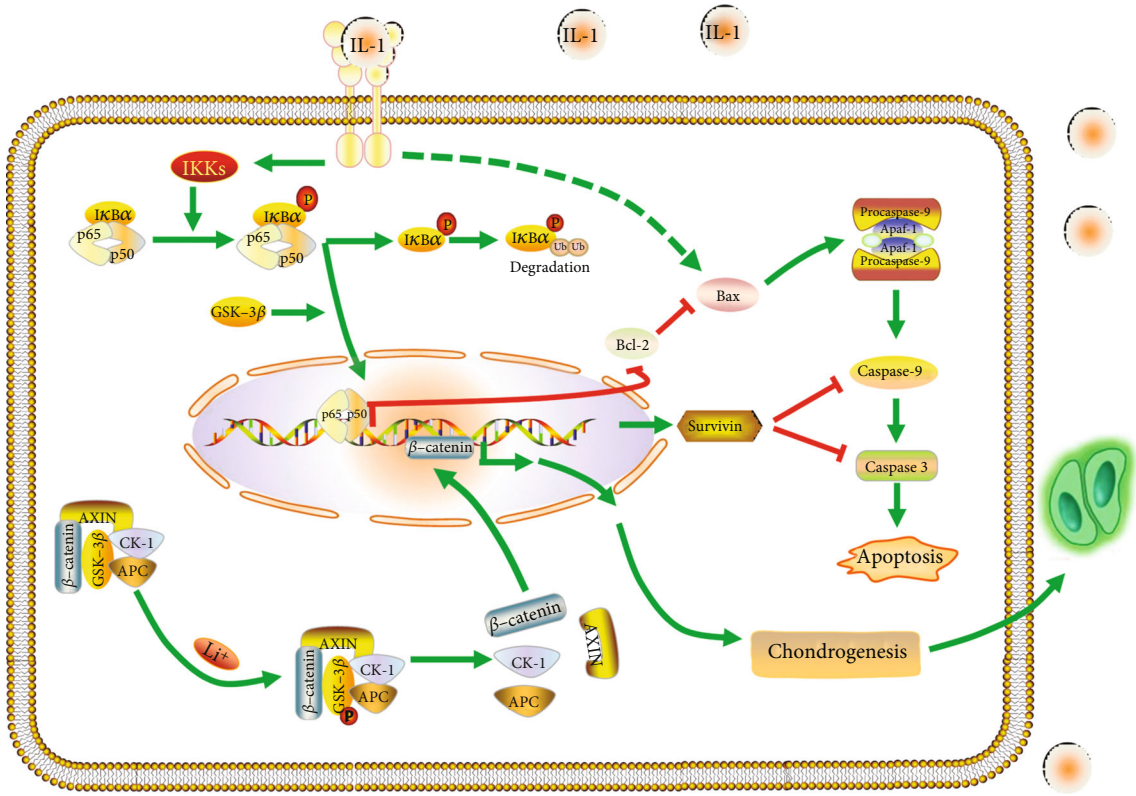


Figure 7: Schematic diagram of the mechanisms. GSK-3β regulates the chondrogenesis via NF-κB signaling and Wnt/β-catenin signaling and possibly mediates the cross-reaction between NF-κB and β-catenin in the nucleus.

that IL-1β suppresses the chondrogenesis ability and cartilage matrix synthesis ability of BMSCs and also induces BMSCs apoptosis during the process of chondrogenesis [13, 17–20]. It has been shown that GSK-3β is extensively involved in the regulation of glucose metabolism, cell proliferation, differentiation, migration, and apoptosis [21–25] and plays a key role in chondrogenic differentiation [26]. In this study, we found GSK-3β exacerbated the effects detected by IL-1β stimulation, which could be reversed by LiCl.

We further investigated the molecular mechanisms of GSK-3β affecting chondroblast differentiation and apoptosis in an inflammatory microenvironment. Previous studies have confirmed the involvement of GSK-3β in the regulation of NF-κB activation [7, 8]. Therefore, the regulation of NF-κB signaling pathway by GSK-3β was first detected. It was found that GSK-3β could activate NF-κB signaling pathway, and only NF-κB phosphorylation was reversed by LiCl. Similarly, Schwabe and Brenner reported that treatment of TNF-α-stimulated cells with LiCl resulted in a decrease of the NF-κB-dependent gene transcription, but did not affect IκBα degradation or IKK activity [27]. Meanwhile, in our study, GSK-3β promoted nuclear translocation of NF-κB p65, which was attenuated by inhibition of GSK-3β activity (LiCl) or NF-κB translocation inhibitor (SN50).

A large number of studies have shown that the Wnt/β-catenin signaling pathway plays an essential role in regulating cell proliferation and differentiation [28–31], and GSK-3β is the crucial regulator of the Wnt/β-catenin signaling pathway [30]. Next, we examined the regulation of GSK-3β on Wnt/β-catenin signaling pathway. As expected, GSK-3β upregulated the phosphorylation of β-catenin, while LiCl inhibited the activity of GSK-3β and thus downregulated the phosphorylation of β-catenin. Furthermore, we detected the expression of β-catenin in the nucleus and found that it was significantly decreased after GSK-3β stimulation. This may be due to the phosphorylation of β-catenin leading to its degradation, thereby reducing the nuclear translocation [32]. Importantly, the expression of β-catenin in the nucleus was increased when nuclear translocation of NF-κB p65 was specifically inhibited by SN50, suggesting a cross-reaction between NF-κB and β-catenin in the nucleus.

In summary, our study confirmed the role of GSK-3β in chondrogenic differentiation of BMSCs in IL 1β-induced inflammatory microenvironment. Additionally, our research provides a new insight into the molecular mechanisms that GSK-3β regulates the chondrogenesis via NF-κB and Wnt/β-catenin signaling pathways and mediates the cross-reaction between NF-κB and β-catenin in the nucleus (Figure 7).

Data Availability

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Zhenggang Wang contributed with the data curation (lead), formal analysis (equal), investigation (lead), methodology (equal), visualization (equal), writing original draft (equal), writing-review, and editing (equal). Zhiyi He contributed with the data curation (equal), formal analysis (equal), investigation (equal), methodology (lead), writing-review, and editing (equal). Weikai Zhang contributed with the formal analysis (equal), investigation (equal), and methodology (supporting). Shuang Liang contributed with the data curation (equal) and methodology (equal). Kun Chen contributed with the formal analysis (equal), writing-review, and editing (equal). Shimeng Xu contributed with the methodology (equal) and validation (equal). Ying Zhang contributed with the supervision (lead), funding acquisition (equal), project administration (equal), writing-review and editing (equal). Peng Cheng contributed with the conceptualization (lead), funding acquisition (lead), project administration (lead), writing-review, and editing (equal). Zhenggang Wang and Zhiyi He contributed equally to this work and share first authorship.

Acknowledgments

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Review Article

The Influence of Different Modes of Exercise on Healthy and Injured Tendons

Kaiyong Wang[1] **and Linlin Zhao**[2]

[1]*Guangdong University of Finance & Economics, Guangzhou, 510320 Guangdong, China*
[2]*School of Kinesiology, Shanghai University of Sport, Shanghai 200438, China*

Correspondence should be addressed to Linlin Zhao; linlinzhao666@163.com

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Tendons are essential components of the musculoskeletal system that links the skeletal muscle to the skeleton. This dense connective tissue exhibits great plasticity. Therefore, research on the influence of types of exercise, including acute and long-term training, on the structural and mechanical properties of tendons in athletic and sedentary populations is of critical importance in the design of scientific-based exercise plans and effective tendinopathy treatment. Here, we review recent studies on the relationship between exercise and tendon health and tendinopathy repair to provide a general understanding of how exercise may reshape tendons.

1. Introduction

Tendon injury, especially patellar and Achilles tendon injuries, exhibits a high prevalence in professional athletes and impacts sports performance [1]. In sedentary populations and those who undergo repetitive overload of forearms during work, microruptures induced by acute mechanical stimulation and the subsequent unsolved chronic inflammation cause sporadic pain and disability [2]. Exercise has a close relationship with tendon homeostasis and injury repair. Proper exercise training may improve the mechanical function of tendons, while acute excessive loading poses a threat to tissue integrity. With increased understanding of tendon tissue characteristics, more effective exercise plan could be designed for different populations. Regarding the influence of exercise on tendons, it should be clearly noted that the modes, intensity, and frequency of exercise are all key parameters that need to be taken into account because different exercise executions involve various components of the musculoskeletal system and pose distinct challenges to energy metabolism and extracellular matrix (ECM) remodelling. On the other hand, the tendons in different locations of the body may experience force transmission to variable extents. Apparently, tendons that are mostly used and injured, for instance, the patellar tendon and Achilles tendon, attract most of the attention of researchers.

To better monitor and reflect the situation of tendons, many methodologies have been developed and utilized. The mechanical properties of the tendon including stiffness, tensile strength, cross-sectional area (CSA), slack length, and elasticity are assessed by protocols based on ultrasound [3]. In noninvasive ultrasound elastography, an ultrasound probe emitting external compression of the tissue is used to attain real-time measurement of the mechanical properties. Ultrasound has been applied in the in vivo assessment of different tendons [4, 5]. Moreover, measurement of the biomechanical properties of tendon grafts serves as a significant reference for surgical treatment with transplantation. Classical tensile testing machines and alternative video tracing measurements with more accuracy realized by digital cameras are applied in the examination of the mechanical properties of tendon grafts [6].

Different exercise styles often affect the rehabilitation care of patients with tendon injury. Previous studies have shown that patients with tendon injury usually progress from low-intensity exercise to high-intensity exercise, with

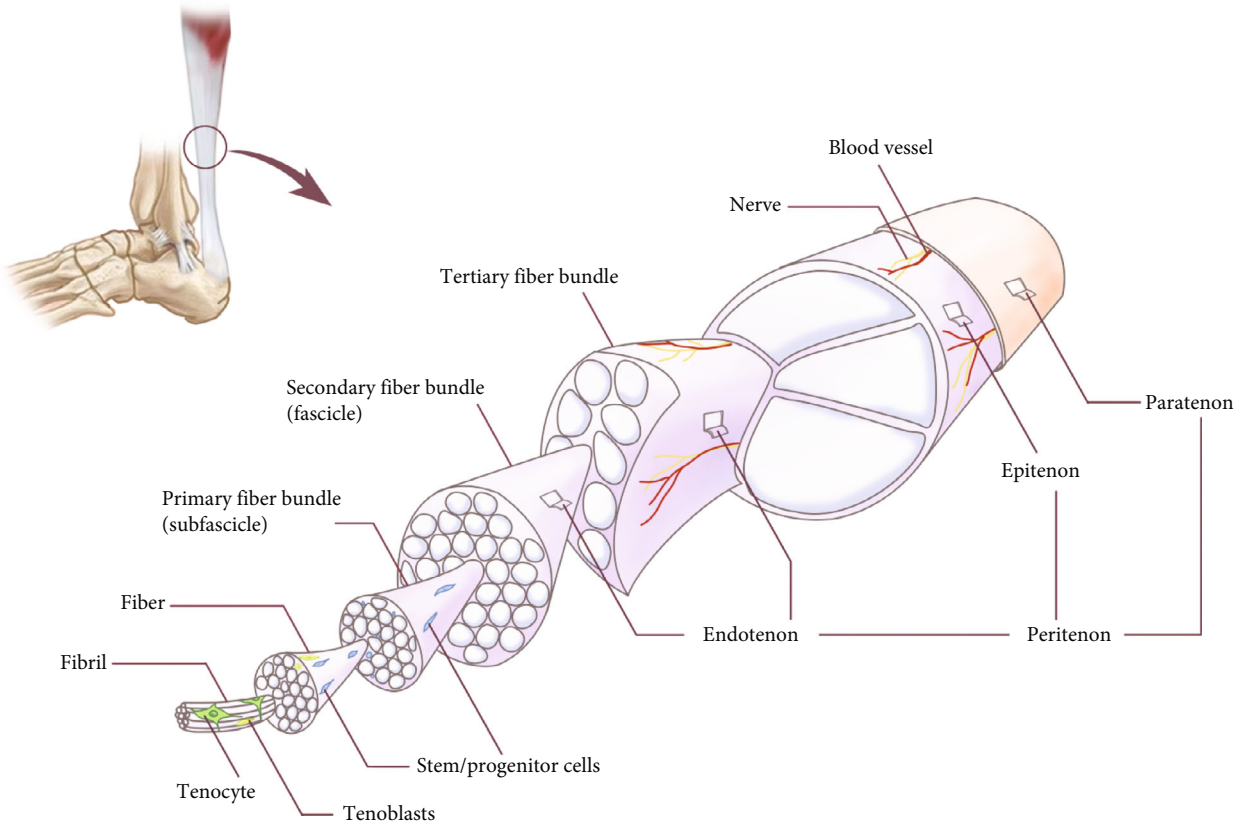

Figure 1: The anatomy structure of tendon. The cell composition including tenocytes, tenoblasts, and tendon-derived stem cells (TDSCs) and the fibre organization of tendon and the enveloping structure are presented.

increasing tendon load that patients must endure before fully resuming high-demand activities [7]. While tendon loading has been shown to improve patient symptoms, normalize tendon structure, and optimize functional performance, both acute and chronic Achilles tendon injuries have negative consequences when overloading or underloading [8]. Therefore, research on the influence of types of exercise, including acute and long-term training, on the structural and mechanical properties of tendons in athletic and sedentary populations is of critical importance in the design of scientific-based exercise plans and effective tendinopathy treatment. Here, we review recent studies on the relationship between exercise and tendon health and tendinopathy repair to provide a general understanding of how exercise may reshape tendons.

2. The Physiology and Pathology of Tendon

2.1. The Anatomical Structure of the Tendon. Before investigating the relationship between exercise and tendon tissue, a comprehensive understanding of the physiology and pathology of tendons is necessary (Figure 1). Located in many parts of the body, tendons are a crucial component in the locomotion system, which connects skeletal muscle and bone to play a mechanical stimulation transition role. In addition to force transmission, the tendon also absorbs shock and stores energy. Frequent use and heavy loading reduce the energy the tendon absorbs but increase the adaptivity to heavy loading, whereas a low rate of loading increases the viscosity of the tendon and the energy it absorbs. Given its abundance in ECM, normal tendon function largely depends on the homeostatic metabolism of these extracellular constituents. In addition to the ECM, the process of tendon development and regeneration is tightly regulated by transcription factors and growth factors belonging to different signalling pathways [9]. In the three germ layers during embryonic development, the origination of tendon includes ectoderm and mesoderm. Craniofacial tendons develop from neural crest cells of the ectoderm [10]. While tendons in the limb bud derived from the tendon progenitor cells in mesoderm, whose development are regulated by the signals of ectoderm and bone morphogenetic protein (BMP) signaling [11].

Linking the skeletal muscle to the bone, the tendon transmits the mechanical force from the skeletal muscle to the bone, enabling locomotion. Similar to the structure of the skeletal muscle, the fibrils in tendons have a hierarchical relationship. The fibrils composed of type I collagen in a triple-helical form fibre, then fascicles, and finally the tendon [12]. To form fibrils, tropocollagen is synthesized in fibroblasts secreted and cleaved extracellularly to become collagen. As the highest hierarchy in the tissue, fascicles are enfolded by a mesh of loose connective tissue, namely, endotenon. The endotenon structure enables the compartmentalization of

fibre so that other components including blood vessels, nerves, and lymphatics can infiltrate and nurture the fibre. The epitenon, surrounding the whole tendon, is continuous throughout the inner surface with the endotenon. In the outermost layer, fatty, the areolar tissue is penetrated by nerves and vessels and surrounded by paratenon, which allows the tendon to move freely in the surrounding tissues without friction. The thickness and organization of the fibril as well as the number, size, and orientation of the collagen fibre coordinately determine the strength and viscoelasticity of the tendon. In addition to type I collagen, the other ECM components in the fascicles include proteoglycans, glycoproteins, elastin, and many other types of collagens, which undergo reprogramming and remodelling in the context of long-term mechanical stimulation or injury healing [13]. As the cell type that dominates in mature tendon tissues, tenocytes take a great responsibility in ECM production, maintenance, and remodelling. It is through regulating the extracellular environment that tenocytes sense and respond to mechanical stimulation [14]. During degradation, the enzymes for protein cleavage secreted out of the cells so that the resulted digestion products can be phagocytosed by tenocytes. These recycled collagen fibrils may be used for new ECM formation intracellularly [15].

Away from the middle part of the tendon, the heterogeneity increases given the formation of junctions that link the tendon to the skeletal muscle and the skeleton, namely, the myotendinous junction and osteotendinous junction, respectively. Between the muscle and the tendon, the myotendinous junction receives stress during contractile force transmission, where the fibre from the tendon inserts into the muscle body to increase the stability of the structure. The fibrocartilaginous tissue that connects the tendon and the skeleton is also called an enthesis. A large amount of type II collagen produced by chondrocyte-like cells in this area shapes the different mechanical properties and pathogenesis of enthesis and tendon, although injuries occurring in both areas are not easily repaired completely.

2.2. Blood Vessels and Nerves in the Tendon. Although the situations are not all the same in all the tendons in the body, generally, the tendons are relatively poorly vascularized with more dependency on synovial fluid diffusion to provide nutrition. The myotendinous junction, enthesis, and surrounding connective tissue such as the paratenon serve as the origin of blood supply for the tendon [16]. Blood vessels from the perimysium, periosteum, paratenon, and mesotendon penetrated the endotenon and epitenon into the fibre and those blood vessels from the paratenon predominating in the middle part of the tendon. As a crucial source of nutrition and an approach to metabolite exchange, the blood vessel pattern changes when tendon injury results from acute friction, rupture, torsion, or compression, especially in frequently used tendons such as Achilles tendons. The phenotypes are controversial. Specifically, hypovascularity was found in some degenerated or ruptured tendons, whereas large blood vessels were found in some tendinopathy cases. Of note, blood vessels are not always a good sign for tendon injury healing. The paratenon is also an origin of sensory nerves for the tendons. The nerve plexuses penetrate the epitenon and branch inside the tendon fibre [17]. The innervation of tendon tissue proper is described as relatively scarce [18].

2.3. Cell Composition of Tendon. Mature tendons are composed of dense connective tissue that is hypocellular, with most of the cells in the tissue being active and being highly proliferative and fibroblast-like tenoblasts and terminally differentiated tenocytes. The tenocytes are spindle-shaped flat cells lying in rows between collagen fibres, forming an exquisite three-dimensional network in the ECM to maintain cell–cell communication [19]. The highly developed rough endoplasmic reticulum in the tenoblasts allows the efficient production of collagen and other ECM. Tenocytes are tenospecific fibroblasts responsible for the production, maintenance, repair, and modification of the ECM [20]. Scleraxis (Scx) and tenomodulin (TNMD), as relatively specific molecular markers of tendons, are often used for the identification of tendon cells, and these proteins play a central role in the development and maturation of tendons [21]. Cserjesi et al. discovered Scx in 1995 using the yeast two-hybrid system for cell-type-specific proteins. Scx is expressed in early embryonic development and regulates tendon maturation [20]. Tenomodulin is a class of type II transmembrane proteins first reported by Shukunami et al. that can regulate the proliferation of tendinocytes and the maturation of collagen fibre [20, 22]. With the explosive-increase in the stem cell research field and proposed bioengineering tissue repair concepts, the search for a cell population with excellent regenerative capability in tendon tissue is ongoing. The identification of tendon-derived stem cells (TDSCs) in animal models and humans is necessary for the elucidation of the cell hierarchy development trajectory of tendons and the identification of new cell sources for bioengineering approaches in tendon injury treatment.

TDSCs were first isolated from human hamstring and mouse patellar tendons in 2007, after which TDSCs were identified in many other animals including rats and rabbits. TDSCs express various markers, including octamer-binding transcription factor-4 (Oct-4), Nanog, nucleostemin (NS), stage-specific embryonic antigen (SSEA)-4, c-Myc, and SRY-box transcription factor (Sox) 2 [23]. Here, we focus on the relationship between exercise interference and tendons, and more information about the labelling of the TDSCs can be found elsewhere.

2.4. Tendon Mechanobiology. Macroscopically, exercise stimulates the tendon mechanistically. Upon activation of molecular signalling pathways, mechanical stimulation signals are conveyed by the ECM deep into the cell and converted to intracellular biological signals by mechanosensitive receptors in the cell plasma membrane. Investigations of the response of tendon cells to mechanical stimulation and the underlying mechanism are of significance for guiding exercise interference in tendon remodelling and tendinopathy treatment. Conversely, the observation and summary of the adaptation of tendons to mechanical loading would enlighten research on the pathogenesis of tendinopathy. With the development of different modes of mechanical

stimulation including compression and tension and bioengineering methods to construct cultured tendon tissue, an increasing number of molecules and related signalling pathways involved in the mechanical regulation of the tendon have been reported.

Composed of tendon cells, strong collagen fibre, and diverse noncollagen ECM, tendons are dynamic responders to frequent mechanical loads. ECM remodelling during this process influences cell function. The secretion and degradation of the ECM in tendons change sensitively after mechanical stimulation to adapt to the environment and maintain homeostasis. Appropriate mechanical force from the structures around the tendon is necessary for the development and maturation of the tendon. Among the molecules that participate in the regulation of tendon dynamics, scleraxis (Scx) has been studied most. This transcription factor is expressed specifically in progenitors and cells in all tendons. Scx deficiency in mice results in limited use of all paws and back muscles and a complete inability to move the tail [24]. Fibroblast growth factor (FGF) in the MAPK/ERK signalling pathway and transforming growth factor-beta (TGFβ) in the SMAD2/3 signalling pathway are reported to regulate the expression of Scx [25, 26]. During injury repair, numerous growth factors and cytokines are expressed and secreted to fulfil the proliferation and remodelling of tendons. Crucial transcription factors including Scx and Mohawk (Mkx) are upregulated after injury [27]. The upregulation of the transcription factors paves the way for alteration of the extracellular environment. Early growth response protein 1 (EGR1) is required for the transcription of Col1a1 [28]. Fibroblast growth factors (FGFs) also promote the production of collagens [29]. TGFβ signalling functions in ECM organization induced tenocyte morphogenesis and the formation of myotendinous junctions [19, 30]. In addition to molecules that are directly related to tendon cells, other cell types such as immune cells and endothelial cells and their corresponding factors, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), are critical to confer recovery ability to the tendon [31, 32]. Further mechanistic research would reveal more unknown factors with the potential to be treatment targets to develop better strategies for tendon injury repair.

3. Exercise and Tendons

3.1. Acute Exercise and Tendons. The immediate effect of a certain mode of exercise on tendons is worthy of exploration for several reasons. Acute exercise with excessive dose and intensity may expose the tendon on the risk of strain injury, partial to complete rupture, and structural destruction of the myotendinous junction [33, 34]. Measurements and analysis of the morphological and mechanical property changes in tendon tissue after acute exercise serve as the basis to understand the mechanism of acute tendon injury and thus to develop effective treatment intervention for exercise and nonexercise populations. It is also important to note that the same exercise mode and dose may have different effects on tendons in healthy and diseased situations due to their tolerance and the capacity to restore after mechanical loading. Furthermore, the accumulation of knowledge of tendon mechanical properties is valuable for the prevention of acute tendon injury and consequential disability and pain.

The stiffness of the Achilles tendon is reduced after maximal voluntary contractions [35, 36]. However, during exercise in the stretch shortening cycle mode, including running and hopping, the stiffness of the Achilles tendon did not significantly change [37]. After acute eccentric exercise, the diameter of the Achilles tendon is reduced significantly [38, 39]. The Achilles tendons of 14 participants who underwent acute eccentric heel drop exercise exhibit significantly increased free tendon length and strain [40]. Generally, the Achilles tendon responds to prolonged stretching and repeated maximal isometric contractions of the triceps surae with transient reduced stiffness and hysteresis, whereas the morphological and mechanical properties do not seem to change after stretch-shortening cycle exercise, such as treadmill running and hopping [41]. It is concerning that the reduced stiffness of the tendon resulting from acute overload may increase the probability of tendon injury.

In addition to morphological analysis, for in-depth knowledge of the alterations in tendons after acute exercise, protein synthesis was examined in tendons. To assess protein synthesis in human subjects, isotope labelling through intravenous injection was employed, and the labelling of target proteins was quantified by gas chromatography–mass spectrometry for quantification. The study showed a rapid increase in collagen synthesis in the patellar tendon after strenuous exercise in the young male volunteers, and the levels peaked at 24 hours post exercise [42]. Another assessment of the expression of extracellular matrix components and related factors revealed no change in the mRNA levels of collagens and noncollagenous matrix proteins, whereas mRNA expression levels of insulin-like growth factor IEa (IGF-IEa) decreased. The biopsies in this research were collected from 31 healthy young men who performed one-leg kicking at 67% of the maximum workload for 1 hour [43]. Given that an increase in collagen protein was detected in other studies, the lack of a change in mRNA expression may imply that acute exercise with a certain intensity may regulate the cleavage of procollagen and metabolism of extracellular collagen. It would be intriguing to explore the alteration in intracellular and extracellular collagen metabolism after acute exercise.

In an observation of calf Achilles tendon, isolated eccentric and concentric loading with the addition of 20% body weight of the Achilles tendon was exerted by the two limbs separately. Sonograms collected 3-24 hours after exercise showed that both loading conditions resulted in decreased normalized Achilles tendon thickness, whereas eccentric loading induced a significantly greater Achilles tendon thickness than concentric loading. However, the transient change after the two modes of exercise completely recovered during a similar time course [38]. An experiment involving 6 males and 4 females reported that there is no difference in the stiffness of Achilles tendon after a single round of hopping exercise with the assessment technique ultrasonography and MRI, although the maximum tendon force during maximum voluntary contraction was reduced after the

exercise [44]. Although the number of subjects in this report was relatively small, the findings suggested that in healthy individuals and potentially in all cases, a single round of physiological stress level exercise did not result in mechanical fatigue of the Achilles tendon.

Stretching is also necessary to adjust the state of skeletal muscle and tendons before and after exercise. Dissection of the function of stretching in tendon movement is important to obtain a proper warm-up effect before and after relaxation of concentrated mechanical loading. A study with 14 participants (7 men and 7 women) showed that the range of motion and maximum voluntary contraction increased after 5 rounds of 60 seconds of static stretching, whereas the muscle-tendon stiffness decreased immediately but not at 5 or 10 minutes poststretching. The mechanical changes in the muscle-tendon junction were attributed to changes in muscle elongation instead of changes in tendon morphology [45].

Although experimental and clinical data have continued to accumulate since the scientific management of the musculoskeletal system attracted the attention of the public, studies on the relationship between acute exercise and tendon health remain blurry and controversial given variations in the methodologies and exercise modes and doses. Moreover, for exercise and nonexercise populations or young and old populations, the stimulation conditions established during the test should differ, as the tendons of these populations are manipulated in distinct manners via different mechanical properties. Regardless of the measurement used for the tendon itself, real-time, high-resolution observation and reflection of the mechanical state of the myotendinous and osteotendinous junction remain lacking. Acute stretching and shortening of the tendon also place great stress on the junction part of the tendon, and the environment is harsh for repair if rupture occurs due to the diversity and low proliferation activity of the cells. It is important to develop suggestions for injury prevention based on a basic understanding of the mechanical characteristics; moreover, knowledge on the junction is also critical.

3.2. Long-Term Exercise and Tendons. Compared with a single bout of exercise, long-term training with a certain frequency is more likely to reshape locomotion system components. In a study involving 40 Chinese subjects 19 to 25 years old who were divided into two groups subject to frequent or infrequent exercise, the mean thickness of the Achilles tendon of subjects undergoing frequent-exercise was significantly greater than that of subjects undergoing infrequent- exercise. The same result of the CSA of the tendon was reported in the dominant ankle [46]. Similarly, increased CSA and stiffness of the tendon were reported after prolonged eccentric training [47, 48]. Collectively, the increased CSA and stiffness of the tendon was regarded as a positive sign because this would allow the tendon to sustain greater mechanical loading and store elastic energy more efficiently. In long term, the morphological and mechanical changes of tendons made by long-term training reduce the stress that the tendon absorbs and decrease the probability of tendon injury when overload occurs. Research comparing of male athletes and Achilles tendon rupture patients through magnetic resonance imaging and maximal isometric plantar flexion force measurements demonstrated that the CSA of tendons normalized by body weight was greater in athletes accepting intermittent high loads, and no obvious structural or loading property differences were noted between the Achilles tendons of rupture patients and the athlete group. The Achilles tendon of rupture patients did not undergo greater force or stress during maximal voluntary isometric plantar flexion than that of the athletes [49]. To investigate the effect of habitual long-term training on human tendons, the researchers assessed the tendon elongation and CSA of the patellar tendon and Achilles tendon of female runners and nonrunners, ($n = 10$). The patellar tendon and Achilles tendon CSA are all comparable in trained and untrained women [50].

The effects of long-term training in a healthy athlete population on tendons may also result in differences in exercise performance. A study of 26 healthy recreational long-distance runners assessed alterations in tendon force and stiffness resulting from long-term resistance training and running economy [51]. The exercise group was subject to an additional a resistance training intervention in their previous running training for 14 weeks, whereas the training of the control group remained the same. Ultrasonography results indicated that the maximum plantar flexion muscle and tendon-aponeurosis stiffness were significantly increased in the exercise group. In the exercise group, better running economy was reflected by reduced oxygen consumption and energy cost.

Along with other components in the musculoskeletal system, the tendon is a highly adaptive tissue with dynamic changes occurring inside after the stimulation of mechanical loading. A meta-analysis focusing on the adaptation of human tendons to mechanical loading reviewed different studies on human tendon adaptation to mechanical loading [52]. Conclusions from such review may serve as an important reference given that these findings provided more statistical evidence for one topic. They found in the reviewed research that the exercise intervention-induced changes in tendon stiffness seem to be more attributed to material instead of morphological properties. In this analysis, the authors demonstrated that loading magnitude is the key element in the loading regimens in contrast to muscle contraction type. The results from this analysis advocate for a high loading intensity and an intervention duration that is longer than 12 weeks for an effective exercise intervention when studying the influence of exercise on tendon properties.

Diverse animal models simulating human exercise were devised to investigate how exercise influences healthy or injured tendons. The horse is a large animal model used to study human tendon physiology and pathology. Research observing two groups of female horses with high or low intensity exercise training for 18 months found that the collagen fibril diameter of the superficial digital flexor tendon decreased in the high intensity group, but no change in collagen content was observed. Compared with the injury-prone equine superficial digital flexor tendon, the rarely injured common digital extensor tendon showed lower water content and higher elastic modulus after long-term,

high-intensity training; however, no signs of degeneration or mechanical property changes were noted in the superficial digital flexor tendon [53]. In one study, 24-month-old rats were divided into three groups based on a treadmill exercise protocol of sedentary, moderate and high intensity for up to 12 months. However, the exercise level did not have a significant effect on the elastic modulus parameter of the tail tendon; however, a decreasing trend was noted at moderate and high intensity compared with the sedentary control group [54]. This result suggested that even long-term exercise was unable to induce a systemic effect on the mechanical properties of old tendons, the structures of which become disorganized during the process of ageing. It is not practical to expect old tendons to exhibit properties of young tendons when exercise is employed as the only intervention after the structure of the tendon has been completely transformed.

Given its regulatory function in fibroblast proliferation and type I collagen synthesis, the TGF-β superfamily is believed to be an essential signalling pathway that participates in the adaptation of tendons to exercise stimulation [55]. Limb formation failure in TGF-β receptor inactivation mice provides strong evidence. Nevertheless, the spatiotemporal control of gene activation and inactivation by genetically modified mouse models is urgently needed to investigate the function of TGF-β function in adult tendons during types of loading interventions.

A common understanding of the mechanism in tendon physiology and pathology depends on the uniformity of the criteria employed in the research methodology. A myriad factors of factors should be taken into account when reviewing different research results given that the assessment approach and resolution, the exercise dose and intensity, the race, age, and gender of the subjects and many other factors may lead to variable conclusions. It was even suggested by some scientists that conditioning exercises should be performed to standardize the load history of tendons before in vivo sonographic measurements of tendon thickness. According to their findings from 30 healthy male participants, conditioning of the Achilles tendon via resistive ankle exercise induced alterations in tendon structure that improved correlations between tendon thickness and body anthropometry [56]. This detailed exploration of the experimental design provided information for a more scientific protocol to examine the relationship between tendon physiology and exercise.

In the observations of tendon adaptation to acute or habitual mechanical loading, the difference between males and females cannot be neglected. Of note, women are more susceptible to soft tissue injury, but the reason is unknown. It was reported that after a bout of acute exercise, collagen synthesis in tendons of men was upregulated but the increase in tendons of women was less profound or absent. Additionally, the patellar tendon of men increased in size after long-term training, whereas no alterations were noted in women. A lower mechanical strength and reduced rate of connective tissue formation in tendons of women were proposed to explain the increased risk noted in women [57]. Regarding the biological mechanism, ethinyl oestradiol, a synthetic oestrogen, inhibits the acute exercise-induced collagen synthesis in female tendons [58]. More specific mechanisms should be studied to resolve the increased risk of tendon injury in women (Table 1).

4. Exercise and Tendon Injury

Tendon pathologies can be divided into chronic injury and acute injury with partial or complete tendon rupture in different specific locations of the tendon. Tendon repair injury includes three overlapping stages of inflammation, proliferation and remodelling [9]. During an inflammatory phase lasting several days, neutrophils recruit to the site of injury and macrophages clear necrotic debris [59]. About two days after the injury, tendinocytes are recruited to the damaged area and stimulate proliferation. Meanwhile, macrophages transform from phagocytosis to repair and promote fibroblast proliferation and guide ECM remodelling by releasing chemokines and growth factors [60]. One to two months after the injury, the synthesis of type I collagen begins to dominate, and the tendon injury enters the remodelling stage, which lasts for more than a year, and the repaired tissue becomes scarring [61]. Sudden rupture of tendons resulting from inappropriate locomotion system utilization is followed by a cascade of repair featur including inflammation, tendon tissue cell proliferation, and ECM remodelling. Characterized by long-term pain and impaired mobility, chronic tendon injury, which is also known as tendinopathy, is often recognized as resulting from tissue overuse. Chronic inflammation and ECM reprogramming during tendinopathy are closely related to the pathogenesis, but the exact mechanism remains to be explored. Furthermore, the mechanisms underlying the healing and the available interference for tendon degeneration also depend on the elucidation of the pathogenesis of tendon injury.

All the tendons in the body can be injured, among which the tendons that are used more often and perform more mechanical loading are injured more frequently. Triceps tendinopathy occurs frequently in throwing athletes and results from the insertion of the tendon into the olecranon. Patellar injuries, as well as Achilles injuries, are common in athletes involved in repetitive jumping, kicking, and running. Wrist extensor tendinopathy is associated with eccentric loading of the forearm muscle in throwing movements. Overload and repetitive improper employment of the musculoskeletal system and the sequential unsolved chronic inflammation, which impedes the proliferation of cells and remodelling of the tissue, together make the recovery of tendinopathy complicated [62].

Factors, including acute tearing, oxidative damage, and accumulation of microtears, would cause structural destruction of the tendon matrix. The matrix synthesized after injury acts as a template for sequential matrix remodelling. As a result, fibrotic scars with different cell and ECM compositions compared with normal tendon tissue appear. The poor mechanical properties of the scar affect the elongation and energy storage of the tendon [63].

Table 1: The influence of different modes of exercise on tendons.

Classification of exercise	Type of exercise	The influence on the tendon
Acute exercise	Running and hopping	The stiffness of the Achilles tendon did not significantly change
	Acute eccentric exercise	The diameter of the Achilles tendon is reduced significantly
	Acute eccentric heel drop exercise	Free tendon length and strain increased
	One-legged kicking exercise	An increase in collagen protein was detected, no change in the mRNA levels of collagens
	A single bout of hopping exercise	No difference in the stiffness of Achilles tendon
Long-term exercise	Prolonged eccentric training	CSA and stiffness of the tendon increased
	An additional a resistance training intervention after running training (14 weeks)	The maximum plantar flexion muscle and tendon-aponeurosis stiffness were significantly increased
	High intensity exercise (18 months, female horses)	The collagen fibril diameter of the superficial digital flexor tendon decreased
	Moderate and high intensity treadmill exercise (12 months, 24-month-old rats)	Long-term exercise was unable to induce a systemic effect on the mechanical properties of old tendons, the structures of which become disorganized during the process of aging

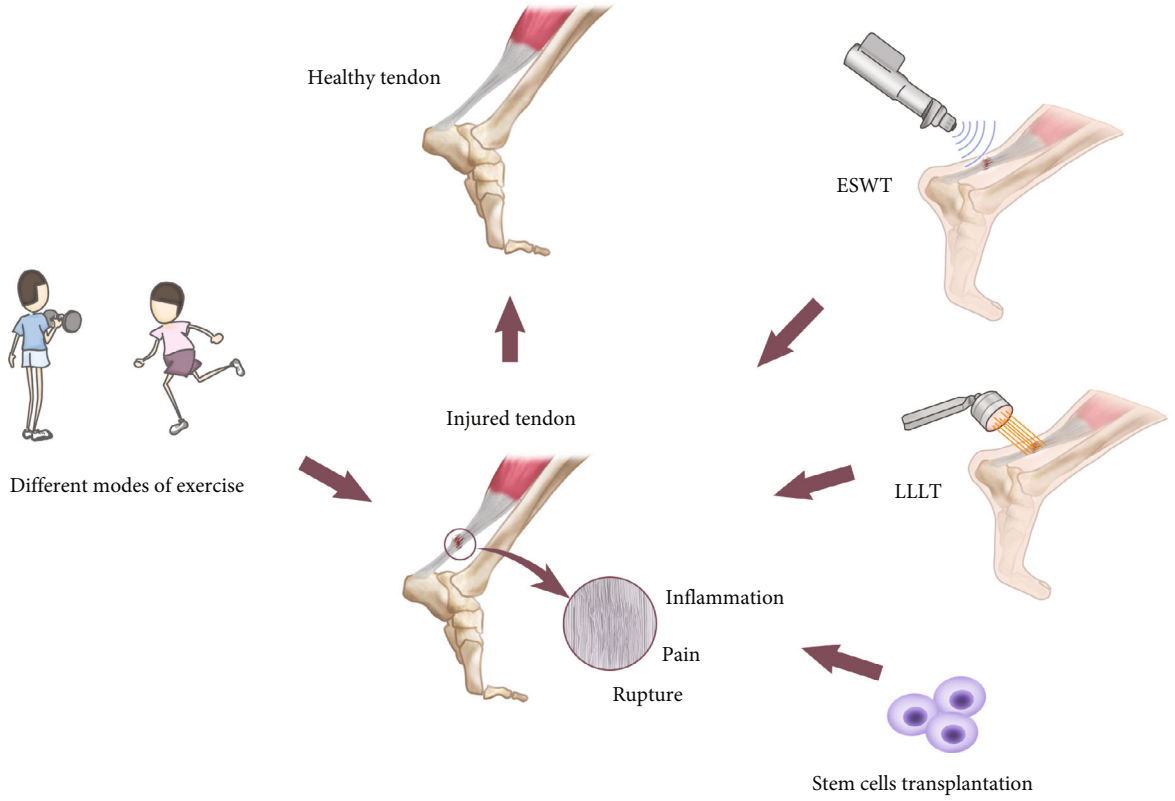

Figure 2: Exercise combined with other approaches in tendon treatment. Different modes of exercise including eccentric exercise and techniques such as extracorporeal shock wave therapy (ESWT), low-level laser therapy (LLLT), and tendon-derived stem cells (TDSCs) transplantation are being applied in the treatment of tendinopathy.

5. Exercise in the Treatment of Tendon Injury and Degeneration

The current treatments for tendon injury mainly include conservative therapy and surgery followed by allogenic transplantation. In the conservative strategies, eccentric exercise therapy and other interventions, including extracorporeal shock wave, ultrasound, and low-intensity lase treatment, are utilized [23] (Figure 2).

A scientifically developed exercise protocol was demonstrated to be effective in Achilles tendon rehabilitation by incrementally increasing the rate and magnitude of tendon loading. To propose guidelines on how to improve the function of tendons, 8 healthy young adults performed a series of

rehabilitation exercise according to the plan. They researchers found that Achilles tendon loading increased when a set of isolated ankle movements or multijoint movements was performed [8]. The finding demonstrates that tendon rehabilitation could be achieved through appropriate exercise given the striking plasticity of tendon tissue. This information has encouraged clinicians and researchers to optimize the exercise training protocol in tendon treatment. Given its potential benefits on the rehabilitation of Achilles tendinopathy, biomechanical research of eccentric exercise on tendon physiology and pathology should be performed to develop an optimal treatment protocol. Real-time recordings of 16 healthy subjects performing one-legged full weight bearing ankle plantar and dorsiflexion exercises revealed no difference in Achilles tendon loads; however, the surface electromyography of the lower leg muscles was reduced [64]. The researchers argued that although the tendon loads are similar, the tendon is vibrated at higher frequencies during the eccentric phase than during the concentric phase. This phenomenon may at least partially explain the effect of eccentric exercise in tendinopathy treatment. In addition to the widely used eccentric exercise, the treatment effect of other types of exercise was also tested. In a clinical study comparing progressive tendon-loading exercise (PTLE) with eccentric exercise therapy (EEP), 76 patellar tendinopathy patients were divided into two groups that received two modes of training [65]. The researchers reported that PTLE resulted in a significantly better clinical outcome after 24 weeks than EET. Such exploration broadens clinicians' horizons and provides superior choice than the conventional eccentric exercise currently recommended.

Extracorporeal shock wave therapy (ESWT) is a physiotherapeutic intervention utilized in tendinopathy treatment. The superiority of the effectiveness of ESWT compared with conservative treatment, such as inflammatory drugs, exercise programs alone, or the use of knee traps in patellar tendinopathy treatment, has been demonstrated [66]. Recently, the influence of ESWT combined with exercise on tendinopathy treatment has been investigated. Thirty-four male athletes suffering from patellar tendinopathy for more than 3 months were divided into an exercise control group who received long-term eccentric exercise (i.e., 12-week single legged decline squat exercise) and a combined group who received a weekly session of ESWT in the initial 6 weeks along with the same exercise plan as the control group. Although a significant reduction in tendon stiffness, an increase in tendon strain and a reduction of in pain intensity were found after eccentric exercise, the addition of ESWT did not seem to have an obvious effect on the clinical outcome [67]. A similar result was found regarding the effect of ESWT on rotator cuff tear repair. Thirty-five patients underwent ESWT for 6 weeks after surgery, whereas the control group did not. The examination included computed tomographic arthrography 6 months after surgery and a minimum one-year follow-up. No significant difference was noted between the two groups [68]. The mechanism was considered to involve the cellular changes induced by mechanotransduction triggered by ESWT [69]. On the other hand, an in vitro positive effect of extracorporeal shock waves on the cell behaviour of tendon cells was demonstrated. In cultured primary human tenocytes, shock wave treatment promoted cell proliferation and collagen synthesis [70]. The differentiation of human tendon-derived stem/progenitor cells was also accelerated by ESWT [71]. Based on in vivo and in vitro results of ESWT research, a deeper understanding of the mechanism of how ESWT influences tendon tissue is expected to facilitate improved clinical guidelines.

Low-level laser therapy (LLLT) has also attracted the attention of scientists in tendon injury repair. In a rat Achilles tendon injury model achieved by surgical hemitransection of the Achilles tendon, different dosages of laser treatment were combined with running exercise for 3 weeks for injury repair. The results showed that rats receiving laser irradiation had less load-relaxation than control rats [72]. During the compensatory overload of the plantar muscle in rats, infrared laser irradiation improved collagen organization in tendons [73]. Combined with adipose-derived mesenchymal stem cell transplantation in rat calcaneal tendon injury, LLLT also hastened collagen organization during tendon repair [74]. Moreover, LLLT functions in different phases in tendon repair including promoting angiogenesis during the inflammatory phase and reducing the inflammatory response during the remodelling phase [75].

In recent decades, burgeoning evidence in animal models and human clinical trials has demonstrated that stem cells derived from different tissues can be utilized in tissue injury repair. The hypocellularity and hypovascularity in the mature tendons makes their self-repair capability very limited. The ECM-enriched tissue environment gives rise to fibrosis and scar tissue with inferior mechanical properties. All these characteristics make the identification and application of TDSCs in the repair of tendon injury urgent and attractive.

Multipotent mesenchymal stem cells (MSCs) are commonly used in tissue regeneration given the abundance of cell resources. The application of MSCs in tendon repair has been tested, and the cells serve to increase early tendon strength and decrease overall healing time [76]. TDSCs are thought to be more suitable for the regeneration of tendons given their high proliferative activity and more mature tendon-like differentiated results. TDSCs promote tendon repair in a rat patellar tendon window defect model and facilitate improved cell alignment and collagen organization [77]. With the assistance of tissue engineering, accurate cell delivery and prolonged retention have been gradually achieved [78]. However, the acquisition of sufficient TDSCs with stable potent properties will still depend on further developed cell culture and augmentation techniques. Although the therapeutic use of TDSCs in tendon treatment is promising due to the potent differentiation capability and plasticity of stem cells, methods require further development given that it is difficult to maintain stem cell properties during the long culture and modification process. Development of an ideal marker combination, standardization of cell culture, amplification and manipulation methods, and the development of transplantation strategies are all noteworthy points.

6. Conclusions

Collectively, we discuss the physiology of tendons and the pathology of tendinopathy. Although the basic anatomical structure of the tendon is well understood, a significant amount of mechanistic information remains unknown. With the exception of tendon cells and the ECM, the composition and dynamics of other cell types, including endothelial cells and pericytes of blood vessels, neurons, and immune cells, in healthy or injured tendon tissue must be further elucidated by newly developed methods, such as intravital confocal microscopy. Acute exercise may lead to transient alterations in collagen synthesis and the mechanical properties of tendons. Acute overloading increases the risk of tendon rupture, whereas proper static stretching prepares the tendon for better mechanical loading performance. The reshaping of tendons based on long-term frequent exercise increases their adaptability to mechanical load stimulation, reduces the probability for injury, and improves better locomotion economy. In investigations on effects of exercise on tendons, the modes and intensity of exercise and the gender of the subjects are all should not be ignored regardless of whether the subjects are athletic or sedentary. Distinct exercise interventions based on the characteristics of the population are recommended for tendon rupture prevention and tendinopathy treatment. Exercise, especially eccentric exercise, is regarded as effective in restoring tendon function. More exercise mode options with logical design are expected. Exercise rehabilitation combined with various technologies, including ESWT, LLLT, tissue engineering, and TDSTs, represents a powerful independent approach or follow-up treatment after surgery.

Conflicts of Interest

All authors state that they have no conflicts of interest interests to report.

Authors' Contributions

Zhao LL gave the brief introduction of this article. Wang KY and Zhao LL were responsible for the manuscript writing. Zhao LL revised the manuscript. All authors approved the final version of this manuscript.

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Research Article

The Effect of Human Bone Marrow Mesenchymal Stem Cell-Derived Exosomes on Cartilage Repair in Rabbits

Hongwei Yang,[1,2,3] **Meng Cong**,[4] **Weixiao Huang**,[4] **Jin Chen**,[5] **Min Zhang**,[4] **Xiaosong Gu**,[4] **Cheng Sun**,[4] **and Huilin Yang**[1,2]

[1]*Department of Orthopedics, The First Affiliated Hospital of Soochow University, Suzhou 215006, China*
[2]*Orthopedic Institute, Soochow University, Suzhou 215007, China*
[3]*Department of Orthopedics, Affiliated Nantong Hospital 3 of Nantong University, Nantong 226001, China*
[4]*Key Laboratory of Neuroregeneration of Jiangsu and Ministry of Education and Co-Innovation Center of Neuroregeneration, Nantong University, 19 Qixiu Road, Nantong 226001, China*
[5]*Department of Orthopedics, Affiliated Hospital of Nantong University, Nantong 226001, China*

Correspondence should be addressed to Cheng Sun; suncheng1975@ntu.edu.cn and Huilin Yang; suzhouspine@163.com

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Mesenchymal stem cells (MSCs) have shown chondroprotective effects in cartilage repair. However, side effects caused by MSC treatment limit their application in clinic. As a cell-free therapy, MSC-derived exosomes (EXOs) have attracted much more attention in recent years. In the present study, we prepared EXOs from human bone marrow mesenchymal stem cells (hBMSCs) and examined their therapeutic potentials in cartilage repair. Our results showed that the prepared extracellular vesicles exhibit classical features of EXOs, such as cup-like shape, around 100 nm diameter, positive protein markers (CD81, TSG101, and Flotillin 1), and ability of internalization. In primary chondrocytes, the treatment of hBMSC-EXOs markedly increases cell viability and proliferation in a dose-dependent manner. Moreover, wound healing assay showed that hBMSC-EXOs accelerate cell migration in primary chondrocytes. JC-1 staining revealed that the mitochondrial membrane potential was enhanced by hBMSC-EXOs, indicating cell apoptosis was decreased in the presence of hBMSC-EXOs. In rabbits with articular cartilage defects, local administration with hBMSC-EXOs facilitates cartilage regeneration as evidenced by gross view and hematoxylin-eosin (H&E) and Saf-O/Fast Green staining. In addition, the International Cartilage Repair Society (ICRS) score was increased by the application of hBMSC-EXOs. Overall, our data indicate that the treatment with hBMSC-EXOs is a suitable cell-free therapy for treating cartilage defects, and these benefits are likely due to improved cell proliferation and migration in chondrocytes.

1. Introduction

Cartilage is a connective tissue with an important role for keeping joints lubricated to ensure smooth movement [1]. Unlike most other tissues, cartilage is composed of gelatinous matrix such as collagen proteins, which were mainly synthesized by chondrocytes. Of note, cartilage has no blood vessels or nerves. Therefore, cartilage regeneration is a tough task due to limited nutrient supply [2]. Cartilage dysfunctions such as defects and injuries often happen that are causative factors for osteoarthritis [3]. Therefore, functional recovery in articular cartilage after injury is a big challenge. Traditional treatments for articular cartilage repair, including nonsteroidal anti-inflammatory drugs, corticoids, acetaminophen, and hyaluronic acid, only ameliorate the symptoms and have marginal effects on cartilage regeneration [4]. Most recently, several strategies were developed for dealing with cartilage defects, including implantation of chondrocytes, osteochondral autograft, and cartilage allograft [5]. However, these mentioned drugs and newly developed strategies still cannot fully resolve cartilage defects in clinic [6]. Hence, alternative strategies aimed at cartilage regeneration are urgently required.

Mesenchymal stem cells (MSCs) have shown promising benefits for cartilage repair due to their potent capacity in differentiation into various types of cells [7]. MSCs could be induced into chondrocyte-like cells to produce extracellular matrix for cartilage regeneration [8]. In addition, MSCs could secrete anti-inflammatory cytokines to modulate immune response, by which a favorable microenvironment was constructed during cartilage repair [9]. To date, MSCs from different sources including bone marrow [10], adipose tissue [11], peripheral blood [12], umbilical cord blood [13], and umbilical cord [14] have been shown promising effects in cartilage tissue engineering. Of them, bone marrow MSCs are considered as the preferred seed cells for treating osteochondral defects due to their advantages in proliferation, chondrogenic differentiation, and easy collection [15]. However, several shortcomings including low survival rate *in vivo* and potential immune rejection impede their application in clinic [16].

Exosomes (EXOs) are small extracellular vesicles with the diameter ranging from 30 to 150 nm, which hold great potentials in translation medicine by delivering functional molecules to treat various disorders [17]. The benefits of MSC-based therapies in tissue repair have been ascribed to the secreted trophic factors, in which EXOs may play a pivotal role. It has been shown that, similar to MSCs, MSC-derived EXOs also have similar effects in tissue repair [18]. Hence, EXOs produced by MSCs, instead of MSCs themselves, were widely used in tissue repair due to the fact that direct use of MSCs may cause side effects such as chromosomal variations and immune rejection [19]. As for cartilage repair, several studies have demonstrated the therapeutic potentials of MSC-derived EXOs. For instance, EXOs from human embryonic MSCs improved cartilage regeneration after injury in rats and micropigs [20, 21]. Articular cartilage repair was enhanced by kartogenin-pretreated infrapatellar fat pad MSC-derived EXOs [22]. EXOs derived from human MSCs promoted cartilage repair and chondrocyte proliferation in osteoarthritis in rats [23]. However, there are several unresolved aspects regarding this newly emerged therapy, including source of MSCs, EXO delivery method and dosage, and the underlying molecular mechanisms.

In this study, therefore, we examined the effects of human bone marrow mesenchymal stem cell- (hBMSC-) derived EXOs on cartilage repair in rabbits. Our results showed that EXOs from hBMSCs greatly improved cartilage repair after injury, and these benefits are likely due to EXOs' induced cell viability, proliferation, and migration in chondrocytes. These data suggest that EXOs derived from hBMSCs hold great therapeutic potentials for treating cartilage dysfunction-associated diseases.

2. Materials and Methods

2.1. Isolation and Culture of hBMSCs. Human bone marrow samples were obtained from Nantong Third People's Hospital. Bone marrow samples were harvested from the iliac crest of six normal human donors (mean age: 38 years old with the range from 33 to 43). The study was approved by the Institutional Review Board of the Affiliated Nantong Hospital 3 of Nantong University (IRB No. EL2022009). All participants gave written informed consent. The procedures for BMSC isolation were described previously [24]. Briefly, 2 ml human bone marrow was mixed with 8 ml alpha-modified Eagle's medium (α-MEM) (HyClone; SH30265.01), in which 10% fetal bovine serum (FBS; Sigma) and 1% penicillin-streptomycin (Beyotime; C0222) were supplemented. The mixture was then added into 100 mm cell culture dish and cultured at 37°C with 5% CO_2 atmosphere. After 4 days, cell culture medium was refreshed to remove nonadherent cells. The remaining attached cells were considered as hBMSCs. Cells were passaged at 80% confluence by adding 0.25% trypsin. Passage 3-4 (P3-P4) cells were used for experiments [25].

2.2. hBMSC-EXO Isolation and Identification. Exosomes (EXOs) were extracted from cell culture supernatant of hBMSCs. For isolating EXOs, culture medium was switched to serum-free medium for 48 h after cells reached 80% confluence. The supernatant was collected and centrifuged at 500g for 10 min to remove cell debris, followed by filtration with a 0.22 μm filter (Millipore). EXOs were isolated using an exoEasy Kit (QIAGEN; 76064) from 15 ml cell culture supernatant according to the manufacturer's protocol. Briefly, filtered supernatant was carefully transferred to a new tube. Then, 1 volume of buffer XBP was added to cleared supernatant. After that, a total of 30 ml of mixture was added onto the exoEasy spin column and centrifuged at 500g for 1 min. After discarding the flow-through, the column was placed back into the same collection tube. The above steps were repeated until the cleared supernatant was no more than 2 ml. Then, 10 ml of buffer XWP was added to the spin column and centrifuged to remove residual buffer at 5000g for 5 min. Transfer the spin column to a fresh collection tube. Add 400 μl buffer XE to the membrane and incubated for 1 min. The eluate was collected by centrifuging at 500g for 5 min. Add the eluate to the spin column and incubated for 1 min again. Finally, collect the eluate after centrifugation at 5000g for 5 min. Carefully resuspend the resulting EXOs in sterilized PBS and store at -80°C until use.

Morphology of EXOs were observed with a transmission electron microscope (TEM, Hitachi). EXO diameter and particle number were analyzed by nanoparticle tracking analysis (NTA, German Particle Metrix) with the software of Zeta View 8.05.04 (German Particle Metrix). Protein concentration was measured using a bicinchoninic acid (BCA) assay kit (Pierce; 23225). Exosomal markers CD81 (Abcam; ab79599), Flotillin 1 (Abcam; ab133497), and tumor susceptibility gene 101 (TSG101; Abcam; ab125011) were examined by western blot analysis [26].

2.3. Rabbit Primary Chondrocyte Culture and Treatment. To isolate primary chondrocytes, 4-week-old New Zealand white rabbits were used. The terminal of tibia and femur was collected for preparing cartilage slices. After 3-time washing in PBS, the slices were incubated with 0.25% trypsin for 0.5 h, and then, 0.2% collagenase II (Sigma-Aldrich; V900892) was added and digestion was performed at 37°C

for 12 h. After digestion, the samples were filtered using a strainer (200-mesh). The resulting filtrate was subjected to 5 min centrifugation at 190g. The residue containing primary chondrocytes was then resuspended in DMEM/F12 medium containing 10% FBS and plated in flasks. P1 chondrocytes were analyzed by immunofluorescence analysis of collagen II. P2 chondrocytes were used for experiments [27]. To evaluate the potential roles of hBMSC-derived EXOs in chondrocytes, the prepared EXOs at different dosages were added into cell culture medium directly.

2.4. Cell Viability Assay. Chondrocytes were seeded in a 96-well plate at 5×10^3 cells/well and cultured in a cell incubator for 6 h. Cells were treated with exosomes at different dosages (2.5×10^8/ml, 5.0×10^8/ml, 1.0×10^9/ml, and 2.0×10^9/ml) for 24 h or 48 h. Cell viability was assayed using a cell counting kit-8 (Dojindo Molecular Technologies; CK04) with the manufacturer's specifications. With a microplate reader (BIOTEK), optical density at 450 nm was measured.

2.5. Cell Proliferation Assay. Cell proliferation was analyzed with a 5-ethynyl-20-deoxy uridine (EdU) labeling kit (Ribobio; C10310-3). 50 μM EdU was added to culture medium, and 2 h incubation was performed. After rinsing two times in PBS, cells were then subjected to 30 min fixation in 4% paraformaldehyde. Glycine (2 mg/ml) was added and incubated for 5 min to remove the aldehyde group. After washing in PBS, cells were incubated with the Apollo staining solution. 30 min postincubation, staining solution was removed and cells were treated with 0.5% Triton X-100 for 10 min. Hoechst 3342 was used to stain the nuclei. Cell images were taken with a fluorescence microscope (Life Technology; EVOS FL Auto). Cell proliferation was analyzed by the percentage of EdU-positive cells.

2.6. Mitochondrial Membrane Potential Measurement. A JC-1 kit (Beyotime; #C2005) was used to detect mitochondrial membrane potential of chondrocytes. In a 24-well plate, cells were seeded and treated with EXOs (1.0×10^9/ml) at 37°C for 24 h or 48 h. Cells were then subjected to JC-1 staining via incubation in the working solution for 20 min, and then, a fluorescence microscope (Life Technology; EVOS FL Auto) was used to observe and take images of cells. Fluorescence intensity was analyzed by the software of ImageJ.

2.7. Scratch Wound Healing Assay. Cell migration was analyzed by wound healing assay [28]. A culture-insert plate (ibidi GmbH) was used in this assay, in which chondrocytes were seeded at 2×10^4 cells per well. 12 h postseeding, the culture insert was removed and nonadherent cells were removed after washing in PBS. Then, cells were incubated with EXOs (1.0×10^9/ml) and photographed at 24 and 48 h post-EXO treatment. With a light microscope, migrated cell numbers were manually counted. For each well, three fields were subjected for counting. The areas were determined using the ImageJ software (National Institutes of Health, USA).

2.8. Exosome Internalization Assay. PKH26 (Sigma-Aldrich; PKH26PCL) was used for labeling EXOs according to the manufacturer's instructions. Excess dye was eliminated by centrifugation at 5000g for 17 min at 4°C using Amicon Ultra-15 tube (Millipore; UFC9050). After 3-time washing in PBS, the pellets were resuspended in PBS and designated as labeled EXOs. For internalization, EXOs were cocultured with rabbit chondrocytes at 1×10^9/ml in serum-free medium at 37°C. 3, 6, 12, 24, 48, and 72 h postincubation, cells were fixed with 4% paraformaldehyde. The nuclei were stained with DAPI, and the cytoskeleton was stained by FITC-Phalloidin (Sigma-Aldrich; P5282). Internalization of EXOs was monitored with a confocal microscope (Zeiss LSM710, Germany).

2.9. Osteochondral Defect Model in Rabbits. Six-month-old male New Zealand white rabbits were provided by the Animal Center of Nantong University. Animals were randomly allocated into four groups: normal group ($n = 6$), PBS group ($n = 6$), low-dosage EXO group ($n = 6$), and high-dosage EXO group ($n = 6$). After anesthetization, rabbits were subjected to surgery to construct cylindrical defects (4 mm × 3 mm; diameter × depth) in the patellar groove of left posterior [29]. To avoid infection, penicillin was given to rabbits at the dosage of 70 mg/kg/day for 3 days via intramuscular injection after the incisions were closed. For pain relief, rabbits received oral meloxicam at the dosage of 0.2 mg/kg/day for 3 days after surgery. One week later, rabbits in the EXO group received 300 μl of 1×10^{10} particles/ml (low dosage) or 5×10^{10} particles/ml (high dosage) by intra-articular injection. Animals in the PBS group were administered 300 μl of PBS. These treatments were performed once a week and lasted for 4 weeks. At the 5th week, rabbit knee joints were collected for further analysis after sacrifice. All animal protocols were approved by the Ethics Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee (Approval ID: SYXK [SU] 2017-0046).

2.10. Histological Staining and Evaluation of Cartilage Repair. Tissues were fixed in 4% paraformaldehyde for 24 h, and then, tissues were subjected to 30-day decalcification in 10% EDTA (pH 7.4). Tissues were then cut into 5 μm thick sections after embedding in paraffin. With 200 μm intervals, the medial and lateral compartments were used for tissue sectioning. After being deparaffinized in xylene, sections were rehydrated using a graded series of ethanol. Thereafter, Safranin O/Fast Green staining (Solarbio; G1371-5) and hematoxylin and eosin staining (H&E) were performed. Sample collection and photographing were described elsewhere [30]. Cartilage repair was evaluated by the International Cartilage Repair Society (ICRS) scoring standard [31].

2.11. Statistical Analysis. The presented data were presented as mean ± SEM from at least three independent experiments. Statistical significance was analyzed using GraphPad Prism software (Version 9.0.0; San Diego, CA, USA). One-way analysis of variance (ANOVA) with Bonferroni's post hoc test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

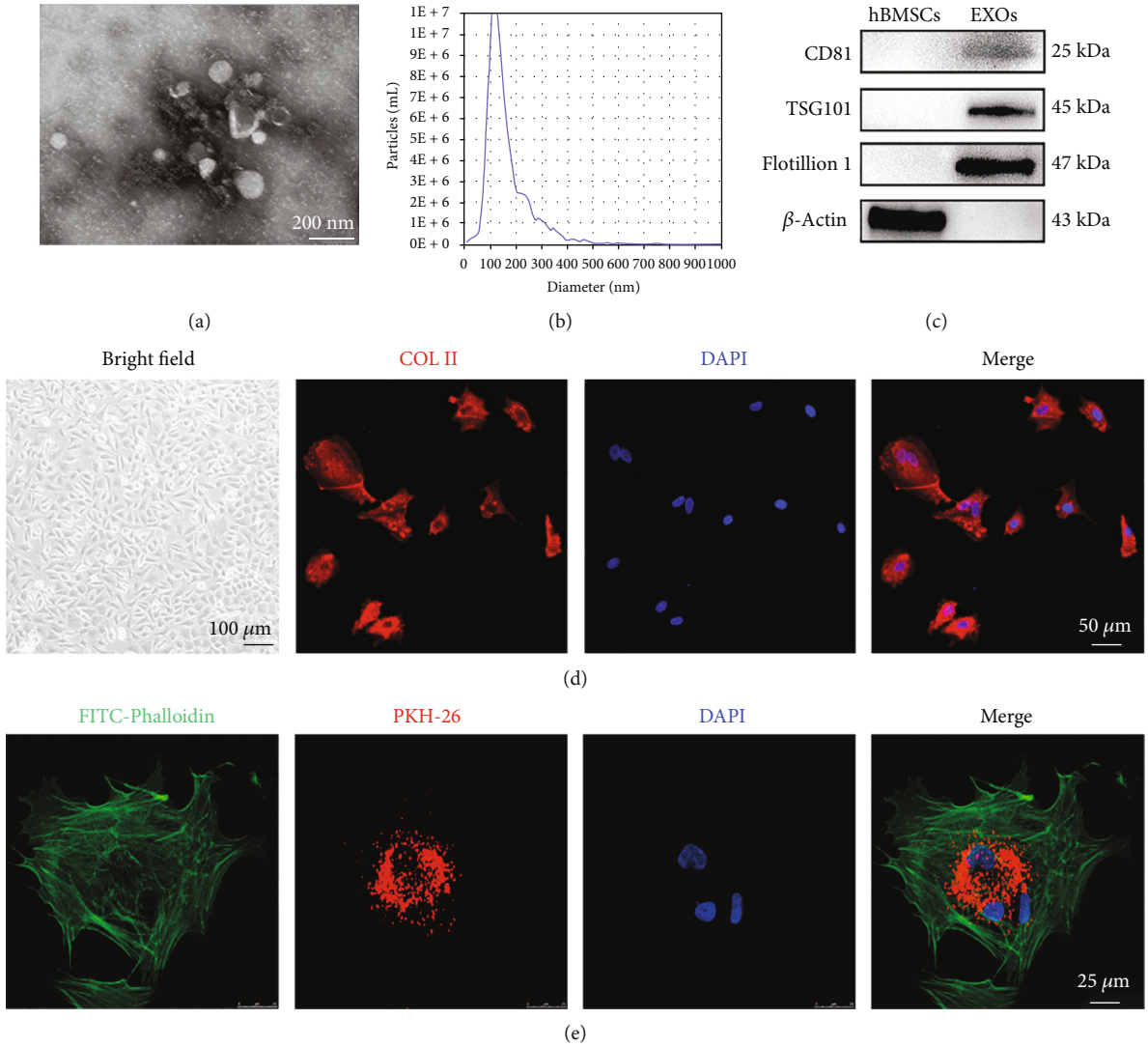


Figure 1: Characterization of human bone marrow stromal cell- (hBMSC-) derived exosomes (EXOs). (a) Representative transmission electron microscope (TEM) images of hBMSC-EXOs (scale bar = 200 nm). (b) Nanoparticle tracking analysis for hBMSC-EXOs. (c) Western blots showing the exosome markers including CD81, TSG101, and Flotillin 1. β-Actin was used as a negative control. (d) Identification of rabbit chondrocytes. Cell morphology was observed at bright field with a microscope (scale bar = 100 μm). Collagen II (COL II; red) was analyzed by immunofluorescence. DAPI (blue) was used to designate the nuclei. Scale bar = 50 μm. (e) Internalization of hBMSC-EXOs in primary rabbit chondrocytes. hBMSC-EXOs were labeled by PKH26 (red) and incubated with primary chondrocytes. FITC-Phalloidin (green) and DAPI (blue) were used to label the cytoskeleton and the nucleus, respectively. Scale bar = 25 μm.

3. Results

3.1. Identification of hBMSCs and hBMSC-derived EXOs. In the present study, hBMSCs were prepared from healthy donors and were used for preparing EXOs. Transmission electron microscope (TEM) images showed that EXOs derived from hBMSCs had a cub-like shape coated with bilayer membranes (Figure 1(a)). Nanoparticle tracking analysis (NTA) revealed that the average diameter of prepared EXOs was 131.2 nm, and the EXO concentration was around 1.2×10^{11} particles/ml (Figure 1(b)). Furthermore, the positive markers of EXOs such as CD81, TSG101, and Flotillin 1 were expressed in hBMSC-EXOs, while β-Actin was only detected in the total cell lysates of hBMSCs (Figure 1(c)). Next, we tested the ability of internalization of these EXOs. To this end, we isolated primary chondrocytes from rabbits, which highly expressed collagen II (Figure 1(d)). As shown in Figure 1(e), EXOs labeled with PKH26 were gathered in rabbit primary chondrocytes according to the confocal microscope images. These data clearly indicate that the prepared extracellular vesicles from hBMSCs are EXOs.

3.2. hBMSC-EXOs Facilitate Cell Proliferation in Chondrocytes. Next, we examined the potential roles of hBMSC-EXOs in primary chondrocytes. The results showed that, after 24 h or 48 h incubation, EXOs markedly promoted cell viability in chondrocytes in a dose-dependent manner (Figure 2(a)). EdU incorporation assay showed that cell proliferation was significantly enhanced by high dose of EXOs (1×10^9/ml) at both 24 h and 48 h (Figures 2(b) and 2(c)).

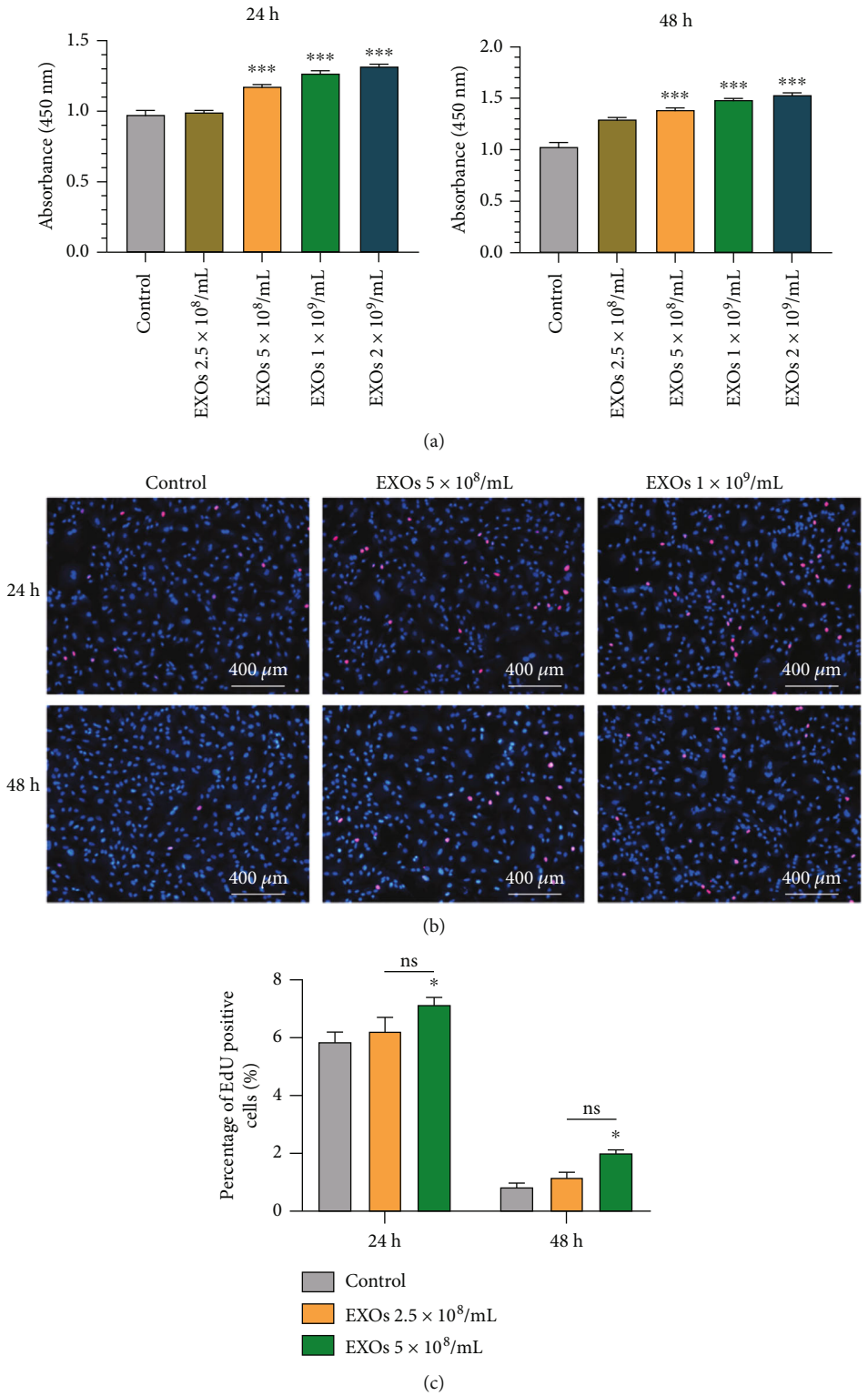
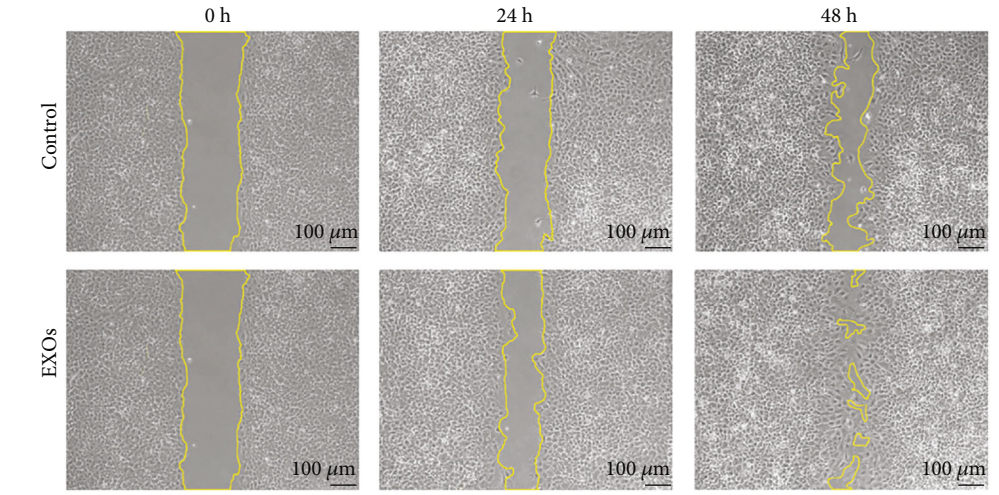
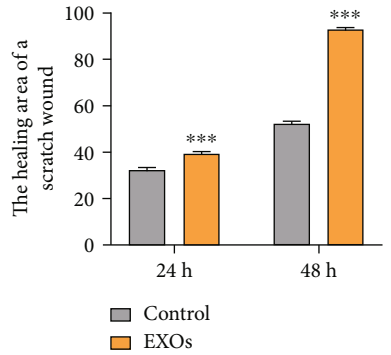


Figure 2: hBMSC-EXOs improve cell viability and proliferation in chondrocytes. Rabbit primary chondrocytes were treated with hBMSC-EXOs at different dosages as indicated. 24 h or 48 h posttreatment, cell proliferation and cell viability were examined. (a) Cell viability was increased by hBMSC-EXOs in chondrocytes. CCK-8 assay was used to analyze cell viability. $n = 3$. (b) Cell proliferation was enhanced by hBMSC-EXOs in chondrocytes. Cell proliferation was assayed by EdU incorporation. $n = 3$. Scale bar = 400 μm. (c) Quantitative analysis for EdU incorporation as shown in (b). Values are presented as mean ± SEM. ns means no significance. $^{*}P < 0.05$ and $^{***}P < 0.001$, versus the control group, one-way ANOVA.



(a)



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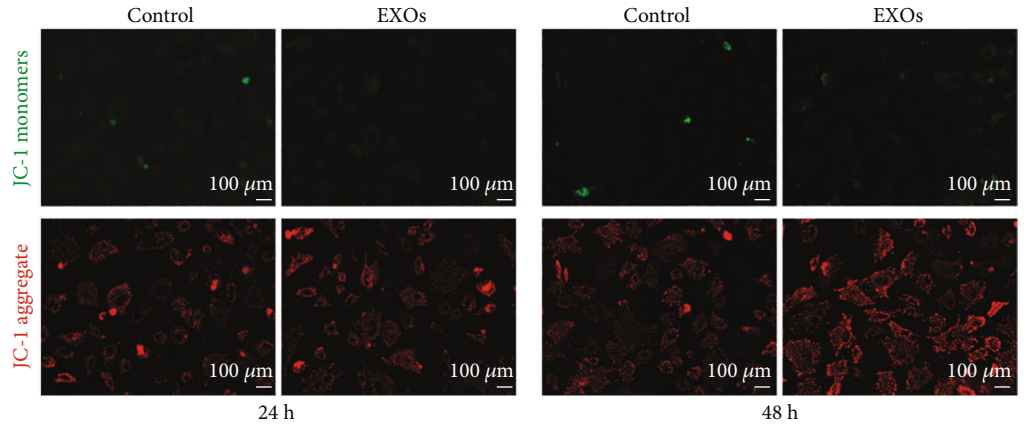

(c)

Figure 3: Continued.

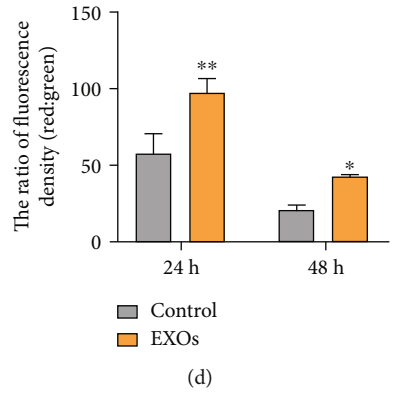

Figure 3: hBMSC-EXOs promote cell migration and mitochondrial function in chondrocytes. Rabbit primary chondrocytes were treated with hBMSC-EXOs at 1.0×10^9/ml for 24 h or 48 h. Cell migration and mitochondrial membrane potential were assayed. (a) hBMSC-EXOs stimulate cell migration. Wound healing assay was used to evaluate cell migration. Scale bar = 100 μm. (b) Quantitative data for wound healing assay as shown in (a). (c) hBMSC-EXOs increase the mitochondrial membrane potential. Representative fluorescence images for JC-1 staining were shown. Scale bar = 100 μm. (d) Quantitative analysis for JC-1 staining as shown in (c). $n = 3$. Values are presented as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, versus the control group, one-way ANOVA.

These data indicate that hBMSC-EXOs are capable of promoting cell viability and proliferation in primary chondrocytes.

3.3. hBMSC-EXOs Enhance Cell Migration and Inhibit Apoptosis in Chondrocytes. Moreover, we also employed wound healing assay to analyze cell migration. The results showed that hBMSC-EXOs accelerated the motility of chondrocytes after 24 h or 48 h incubation (Figures 3(a) and 3(b)). JC-1 is a novel dye for evaluating the mitochondrial membrane potential. A monomer with green fluorescence was presented at low concentrations for this dye. At high concentrations, JC-1 exists as aggregates with an emission maximum at around 590 nm. Our data showed that the treatment of EXOs reduced JC-1 monomers in chondrocyte as evidenced by decreased green fluorescence intensity (Figure 3(c)). Meanwhile, JC-1 aggregates were increased in chondrocytes due to enhanced red fluorescence intensity (Figure 3(c)). As a result, the ratio between red to green fluorescence intensity was improved by hBMSC-EXOs (Figure 3(d)). These data indicate that EXOs derived from hBMSCs have an ability for promoting the cell migration and inhibiting apoptosis in chondrocytes.

3.4. hBMSC-EXOs Promote Cartilage Repair after Injury in Rabbits. The above data showed that hBMSC-EXOs hold benefits in chondrocytes, such as increasing cell viability and proliferation and stimulating the mitochondrial function and cell migration. These observed benefits prompt us to examine whether they have similar functions *in vivo*. To select an appropriate regimen in animal experiments, we first examined the time course effect of exosome internalization in chondrocytes. The endocytosis of EXOs by chondrocytes occurred at 3 h, and thereafter, it was gradually increased till 72 h (Figures 4(a) and 4(b)). Since there is severe overlap of growing cells, we did not extend the test time. Based on these findings, we predicted that the endocytosis of hBMSC-EXOs in chondrocytes may peak at 3-4 days postincubation. Therefore, we adopt the regimen in which hBMSC-EXOs were given to the rabbits once a week. To investigate the role of hBMSC-EXOs in cartilage repair in vivo, we generated osteochondral defect model in rabbits; a cylindrical defect with 4 mm × 3 mm (diameter × depth) was created in the patellar groove. Histological analysis was conducted after a 4-week treatment with hBMSC-EXOs. The whole experimental design is illustrated in Figure 5(a). According to the gross view and H&E staining, the defects still could be seen in the PBS group; however, the treatment of hBMSC-EXOs exhibited visible cartilage repair especially in rabbits treated with higher dosage of EXOs (Figures 5(b) and 5(c)). The Saf-O/Fast Green staining further confirmed that the articular cartilage defects in rabbits were largely improved by hBMSC-EXOs (Figure 6(a)). Meanwhile, we also evaluated cartilage regeneration by using the ICRS visual histological score system. Histological assessment was performed in a blinded manner by the same two independent observers. The scores in the EXO group were markedly improved with comparison to those in the PBS group. Of note, the score was further increased by high dosage of hBMSC-EXOs (Figure 6(b)), which was consistent with the histological staining as mentioned above.

4. Discussion

Cartilage is a flexible tissue, which is mainly composed of water and several types of proteins including proteoglycans, collagens, and noncollagenous proteins. Of note, these specialist proteins are produced by a group of cartilage cells called chondrocytes. Therefore, chondrocytes are extremely important for proper functioning of cartilage. To evaluate the effects of hBMSC-EXOs in cartilage repair, we first analyzed the effects of hBMSC-EXOs on chondrocytes. Our data showed that the cell proliferation and migration in chondrocytes were promoted by hBMSC-EXOs. Chondrocyte proliferation and migration are two essential events for maintaining healthy cartilage. In line with our findings,

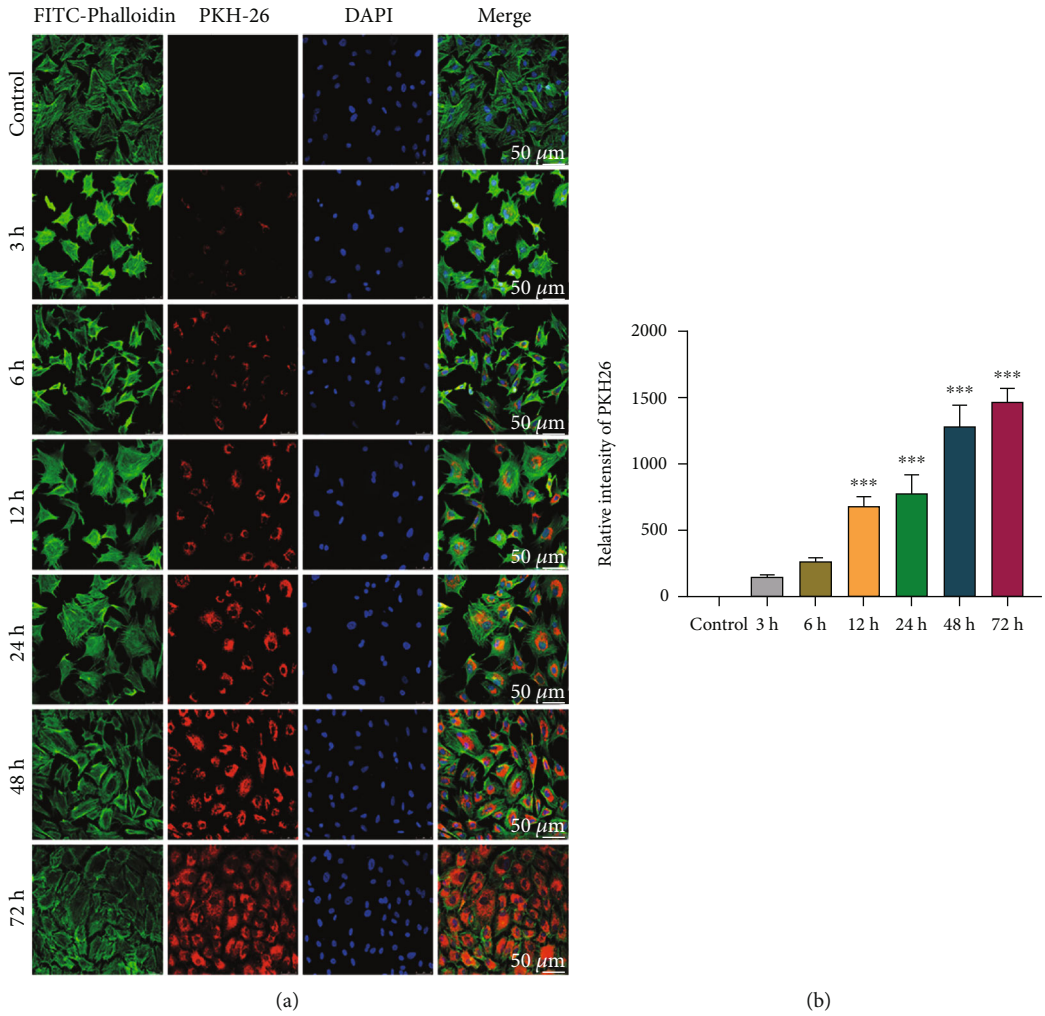

Figure 4: The time course effect of hBMSC-EXO internalization in chondrocytes. (a) EXO internalization was gradually increased along with the time elongation. hBMSC-EXOs were labeled with PKH26 (red) and incubated with primary rabbit chondrocytes at the dosage of 1×10^9/ml. Immunofluorescence staining was performed at different time points as indicated. FITC-Phalloidin (green) and DAPI (blue) were used to label the cytoskeleton and the nucleus, respectively. Scale bar = 50 μm. (b) The fluorescence intensity for PKH26. $n = 3$. Values are presented as mean ± SEM. $^{***}P < 0.001$, versus the control group, one-way ANOVA.

other source EXOs also hold capacity for stimulating cell migration and proliferation in chondrocytes [32–35]. Recently, rat BMSC-derived EXOs have shown to protect chondrocytes from advanced glycation end product-induced damages [36]. EXOs from human urine-derived stem cells (hUSCs) are capable of inducing proliferation and migration in chondrocytes [37]. Moreover, human MSCs-EXOs were found to inhibit autophagy and apoptosis in chondrocytes by activating the axis of PI3K/Akt/mTOR [38]. Mitochondria are the power supply center of the cell, providing ATP in many key biological events, such as cell growth, differentiation, and migration [39]. Defects in mitochondrial function and damages induced by oxidative stress are causative factors for loss of chondrocytes in cartilage defects [40]. In the present study, we treated chondrocytes with hBMSC-EXOs and found that this treatment greatly improves mitochondrial activity. One previous study also showed that EXO-derived MSCs inhibit mitochondrial dysfunction and reduce chondrocyte apoptosis [41].

To date, numerous studies examined the biological functions of EXOs for dealing with various diseases such as cancer, cardiovascular disorders, and neurological syndromes. However, the used dosages of EXOs are inconsistent, which vary with differences in experimental animal species, delivery methods, and kinds of diseases [42]. Several parameters were established and employed for dosing EXOs. For instance, total protein levels, total lipid levels, global RNA contents, and particle numbers are all used for quantifying amount of EXOs [42]. By considering the possibility of contamination of protein and lipids during exosome preparation, particle numbers are widely used for dosing EXOs [42]. In the present study, we also used particle numbers for calculating the dosage of EXOs for treating cartilage defects in rabbits. To select an appropriate dosage, we first examined the effects of EXOs at different dosages on cell viability, proliferation, and migration. Our data showed that 1×10^9 particles/ml is a suitable dosage in cultured primary chondrocytes. Due to reduced bioavailability *in vivo*, a higher dose (1×10^{10} particles/ml or 5×10^{10}

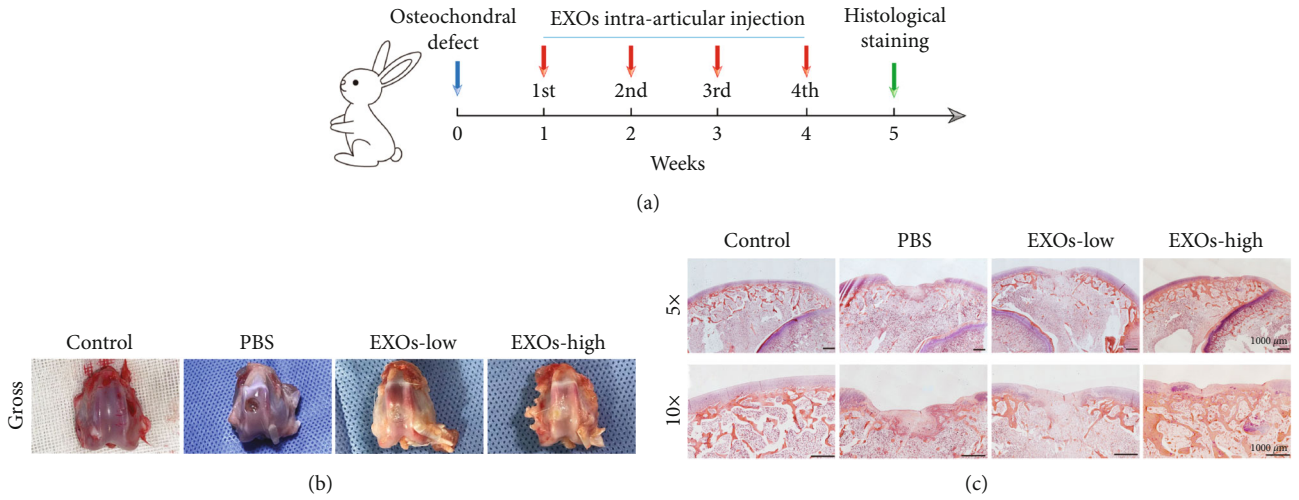

Figure 5: hBMSC-EXOs promote cartilage formation after injury in rabbits. (a) The experimental design. Defects were generated in the articular cartilage of rabbits, and then, hBMSC-EXOs were given to rabbits via intra-articular injection for 4 times. (b) The gross appearance of rabbit articular cartilages. (c) H&E staining. Scale bar = 1000 μm. EXOs-low: 1×10^{10}/ml; EXOs-high: 5×10^{10}/ml.

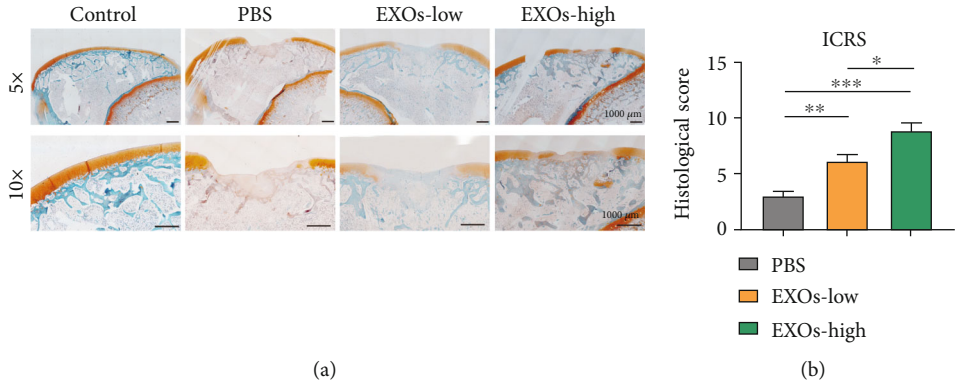

Figure 6: hBMSC-EXOs promote cartilage repair in rabbits. (a) Saf-O/Fast Green staining. Scale bar = 1000 μm. (b) The International Cartilage Repair Society (ICRS) scores. $n = 3$. Values are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, one-way ANOVA. EXOs-low: 1×10^{10}/ml; EXOs-high: 5×10^{10}/ml.

particles/ml; 30 μl for each animal) was adopted for cartilage repair in rabbits, which was comparable to the dose of exosomes for treating osteoarthritis in mice [43]. Moreover, both studies used local administration of EXOs by intra-articular injection. To select an appropriate regimen, we analyzed the time course effects on exosome internalization in rabbit primary chondrocytes. We found that exosome internalization starts at 3 h incubation, and it gradually increases along with the time elongation and peaks at 72 h. Due to severe overlap of cells, we did not observe exosome internalization for longer time. Based upon these data, we conclude that the internalization of hBMSC-EXOs in chondrocytes may peak at 3-4 days after treatment, indicating hBMSC-EXOs could last for 6-8 days. Therefore, we treated rabbits with prepared EXOs once a week for 4 weeks. With this regimen, the prepared hBMSC-EXOs were directly injected into the articular cavity. Functional assays showed that osteochondral defects were largely repaired by hBMSC-EXOs. In line with our findings, several reports also have shown that EXOs from different MSCs have benefits in cartilage repair [20–23, 35].

In general, EXOs act as cargo for delivering various substances such as nucleic acids, proteins, and lipids to recipient cells and thus play their functions [44]. For example, Zhang et al. found that MSC-derived exosomal CD73 stimulates AKT and ERK signaling to increase cell proliferation and infiltration in chondrocytes during cartilage repair [35]. In addition to exosomal proteins, exosomal nucleic acids including miRNAs and long noncoding RNAs (lncRNAs) were extensively examined in cartilage regeneration. Wu et al. showed that miR-100-5p was enriched in MSC-EXOs, which inhibits mTOR signaling and thus attenuates articular injury in osteoarthritis [43]. lncRNA KLF3-AS1 was highly expressed in MSC-EXOs, and it suppresses IL-1β-induced apoptosis in chondrocytes [23, 32]. Based upon these findings, we proposed that hBMSC-EXO-mediated cartilage repair in the present study is likely due to some specific proteins, miRNAs, and/or lncRNAs in EXOs. Nevertheless, lipids and some other metabolites present in hBMSC-EXOs may also play a role in chondrocyte proliferation and extracellular matrix synthesis in cartilage repair. One project

aimed at this topic is ongoing, in which combined technologies including RNA-seq, lipidomics, and proteomics will be employed to explore the underlying mechanisms.

In conclusion, in the present study, we prepared exosomes from hBMSCs to treat primary chondrocytes *in vitro* and rabbits with cartilage defects *in vivo*. Our data showed that hBMSC-EXOs improve cell viability, proliferation, and migration in primary chondrocytes. Meanwhile, hBMSC-EXOs also reduce cell apoptosis *in vitro*. In rabbits with cartilage defects, the application of hBMSC-EXOs largely promotes cartilage repair. These data strongly suggest that hBMSC-EXOs hold great therapeutic potentials for treating cartilage dysfunction-associated diseases. Moreover, these benefits are likely due to improved cellular functions in chondrocytes induced by hBMSC-EXOs.

However, the present study still includes several limitations. First, the observed benefits of hBMSC-EXOs in cartilage repair remain to be validated in more experiments using other animal models. Second, the mechanisms responsible for hBMSC-EXO-induced cartilage repair are still not clear, although we observed hBMSC-EXOs improve cell proliferation and migration in chondrocytes. The involved mechanisms will be elucidated by analyzing the constituents in prepared EXOs and related functional studies.

5. Conclusions

Overall, in the present study, we prepared exosomes from human bone marrow MSCs, which were then used to treat rabbits with osteochondral defects. Our data showed that these prepared exosomes greatly improved cartilage repair after injury. These benefits are likely due to increased cell viability, proliferation, mitochondrial function, and cell migration in chondrocytes induced by exosomes. All these data strongly suggest that exosomes derived from bone marrow MSCs hold great therapeutic potentials for treating cartilage dysfunction-associated diseases such as osteoarthritis and traumatic joint injury.

Data Availability

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

Conflicts of Interest

The authors declared that they have no conflict of interest.

Authors' Contributions

Xiaosong Gu conceived the idea and designed the study. Hongwei Yang, Meng Cong, Weixiao Huang, and Min Zhang carried out cell experiments and acquisition of data. Hongwei Yang and Jin Chen performed osteochondral defect model and histological analysis. Hongwei Yang drafted the manuscript. Cheng Sun and Huilin Yang designed the experiments, analyzed the data, and wrote the manuscript.

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Research Article

Researches on Stem and Progenitor Cells in Intervertebral Discs: An Analysis of the Scientific Landscape

Yunzhong Cheng, Honghao Yang, Yong Hai, and Yuzeng Liu

Department of Orthopedic Surgery, Beijing Chao-Yang Hospital, Capital Medical University, Beijing 100020, China

Correspondence should be addressed to Yong Hai; yong.hai@ccmu.edu.cn and Yuzeng Liu; beijingspine2010@163.com

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Low back pain (LBP) is a common clinical symptom, and the prevalence is ranged from 60% to 70%. With the deepening of basic research, the development of intervertebral disc regeneration-oriented cell therapy, especially stem and progenitor cells therapy, showed good research prospects and was expected to become new methods of treatment for LBP. Our study is aimed at analyzing the scientific output of stem and progenitor cells in intervertebral discs and at driving future research into new publications. Researches focused on this file were searched from the Science Citation Index Expanded (SCI-E) of the Web of Science (WOS) core collection database and were screened according to inclusion criteria. We evaluated and visualized the results, including annual publications, citations, authors, organizations, countries, research directions, funds, and journals by bibliometric website, VOSviewer, and Citespace softwares on May 27, 2022. A total of 450 original articles and reviews were included, and the overall trend of the number of publications rapidly increased. In worldwide, China and the USA were the leading countries for research production. The retrieved 450 publications received 14322 citations, with an average of 31.83 citations and an H-index of 62. The most high-yield author, organization, country, research directions, funds, and journals were Chen QX from Zhejiang University, Zhejiang University, China, Cell Biology, National Natural Science Foundation of China, and Spine, respectively. Keywords cluster analysis showed the research hotspots in the future, including "human intervertebral disc", "adipose-derived mesenchymal stem cell", "intervertebral disc degeneration", "degenerative disc model", "nucleus pulposus regeneration", "human cartilage", "3d culture", "shrinkage-free preparation", and "polylactide disc". Furthermore, with accumulating evidence demonstrating the role of stem and progenitor cells in intervertebral discs, "microenvironment", "activation", "intervertebral disc degeneration", and "oxidative stress" are becoming the research frontiers and trends.

1. Introduction

The intervertebral disc consists of the nucleus pulposus, annulus fibrosus, and cartilage endplates. Proteoglycan in the nucleus pulposus has a large amount of anions and high osmotic pressure, which can absorb water and swell. The expansion converts the compressive load into a tensile effect on the annulus, which acts as a tension "skin" to limit the expansion of the nucleus pulposus [1]. Due to the structural characteristics of the fibrous annulus, its resistance to compression is far less than that of tensile capacity. The fibrous ring structure is easily destroyed when a large compressive load occurred [2]. More importantly, the intervertebral disc itself lacks blood supply; it is very difficult to self-healing once degeneration and damage happened [3]. In recent years, researches on the pathophysiology of intervertebral disc degeneration have opened a new avenue for disc regeneration therapy [4], particularly stem and progenitor cells therapy for intervertebral disc problems.

Bibliometric analysis and visualization are not only more effective methods to assess the thematic development of structural contents. More importantly, it can help researchers to better understand comprehensively about hotspots, frontiers, and trends in particular topic [5–7]. Science Citation Index Expanded (SCI-E) of Web of Science (WOS) Core Collection Database is widely used as an

important tool for scientific statistics and scientific evaluation [8]. Thanks to the quantitative construction of this database and the qualitative contribution of the bibliometrics, most cited publications, top high-yield countries, organizations, authors, research directions, and funds, as well as journals, can be comprehensively analyzed.

However, no bibliometric literature on stem and progenitor cells in intervertebral discs has been researched and reported. Our study aims to draw the outline of the intellectual connections within the dynamic changing of scientific knowledge in the field of stem and progenitor cells in intervertebral discs by making good use of the citation database (SCI-E) and the software tools (https://bibliometric.com/, VOSviewer, and Citespace). These results can benefit scholars by better understanding future research directions and trends.

2. Method

2.1. Data Collecting. The literature data were retrieved through the SCI-E of WOS Core Collection Database in Capital Medical University Library. The search query was"((TI=(Stem Cell OR Progenitor Cell) AND TI=(nucleus pulposus OR disc OR intervertebral discs OR annulus fibrosis OR endplates OR perichondrium)) AND LA=(English) AND DT=(Article or review)". The literature searching was accomplished within a single day to avoid the bias due to database updates on May 27, 2022. The records were exported by "full records and cited references" in plain text file format and tab delimited file format, respectively.

2.2. Bibliometric Analysis. The trends of publications and citations were charted annually. Contribution of all countries by publications was made by a pie chart. A total number of publications and sum of total citations from 1999 to 2022 were obtained. Top 20 Most Cited Articles were recorded and analyzed, including first author, article title, journals of publication, year of publication, total number of citations, and the impact factor of journals. The top 5 records, H-index, total citations, and average citations in terms of authors, organizations, and countries were tabulated directly. The top 5 research directions, funds, and journals with the most publications were meanwhile charted.

The co-authorship analysis of countries on stem and progenitor cells in intervertebral disc degeneration was analyzed by the bibliometric website (https://bibliometric.com/). The co-authorship relations in the analysis units of authors and organizations, the co-citation analysis of references, journals, and authors were all mapped by VOSviewer_1.6.11 software (Nees Jan van Eck and Ludo Waltman, 2019).

Co-citation timeline of references by keywords, keywords clusters on stem and progenitor cells in intervertebral discs, top 25 references with the strongest citation bursts, and top 14 keywords with the strongest citation bursts, as well as details of top 9 clusters, were visualized by CiteSpace_5.8. R3 edition (Chaomei Chen, 2003-2022). The time slicing was selected from January, 1999 to December, 2021. Years per slice was picked by one. The rest of the parameters are chosen by default setting. The reference was selected for co-citation timeline and burst analyses. The keyword was selected for burst analyses, and details of cluster with three different algorithms (LSI, LLR, MI) were exported into a table.

3. Results

3.1. General Information. A total of 450 articles and reviews were retrieved in the SCI-E of WOS Core Collection Database, with a sum of 14322 times cited, average citations of 31.83 per item, and an H-index of 62. Figure 1 showed the annual publications and sum of times cited per year on stem and progenitor cells in intervertebral discs. The year with most publication was 2021 ($n = 52$), and the number of publications showed a fluctuating increase year by year. In addition, the citation started in 2003 ($n = 6$), and the year with most times cited was 2021 ($n = 2098$), and citations increased linearly year by year.

3.2. Publications Distribution in Different Countries of the World. A total of 25 countries were retrieved with publications on stem and progenitor cells in intervertebral discs. China and the USA were in a dominant position, accounting for more than 70% in all over the world (Figure 2(a)). China had contributed 231 articles (51.33%) at the top. The USA is the second contributing country with 94 articles (20.89%), followed by England with 32 articles (7.11%), Japan with 30 articles (6.67%), and Switzerland both with 23 articles (5.11%) (Figure 2(b)). Total times citations of the USA were 4792 at the first, followed by China (4523), Japan (2612), England (1654), and Switzerland (950) (Figure 2(c)). Meanwhile, the H-index of the USA was 37 in the first place, China was the second with 33, Japan (21), England (18), and Switzerland (15) (Figure 2(d)).

3.3. Top 20 Most Cited Articles. A total of 450 articles from Web of Science were collected. Top 20 most cited articles on stem and progenitor cells in intervertebral discs are showed in Table 1, including first author, article title, journals of publication, year of publication, total number of citations, and the impact factor of journals. The total citations of the top 20 articles ranged from 141 to 311. The top article had 311 citations and was published in 2003 by Sakai D [9], followed by Sakai D [10] with 277 citations in 2006 and Sakai D with 267 citations in 2012 [11]. The oldest article was published by Arai F in 2002 [12], and the most recent article in top 20 was published in 2016 by Richardson SM [13]. More importantly, the impact factor of 1 article was more than 20, the impact factor of 6 articles was more than 14, and the impact factor of 10 articles was more than 6.

3.4. Contribution of Authors, Organizations, and Countries. 1909 authors, 532 organizations, and 25 countries contributed to this field, respectively. Table 2 showed that the top author with most publications was Chen QX ($n = 22$) from Zhejiang University and Zhou Y ($n = 22$) from Army Medical University [14, 15], followed by Li FC ($n = 20$) from Zhejiang University [16], Liang CZ ($n = 20$) from Zhejiang University [17], and Li H ($n = 19$) from Shanghai Jiao Tong University [18]. Of the 532 organizations, Zhejiang

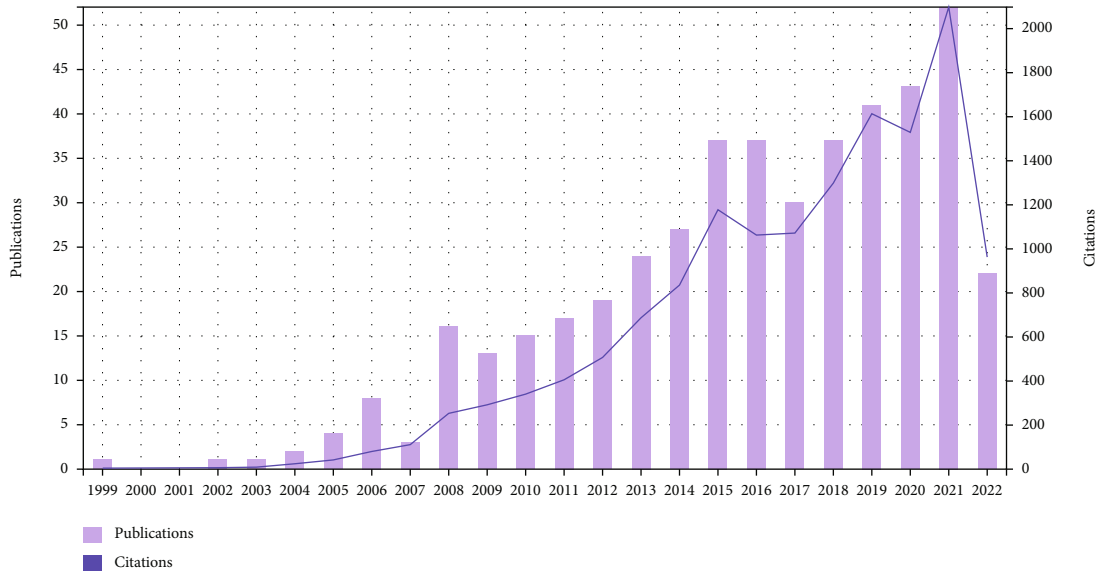


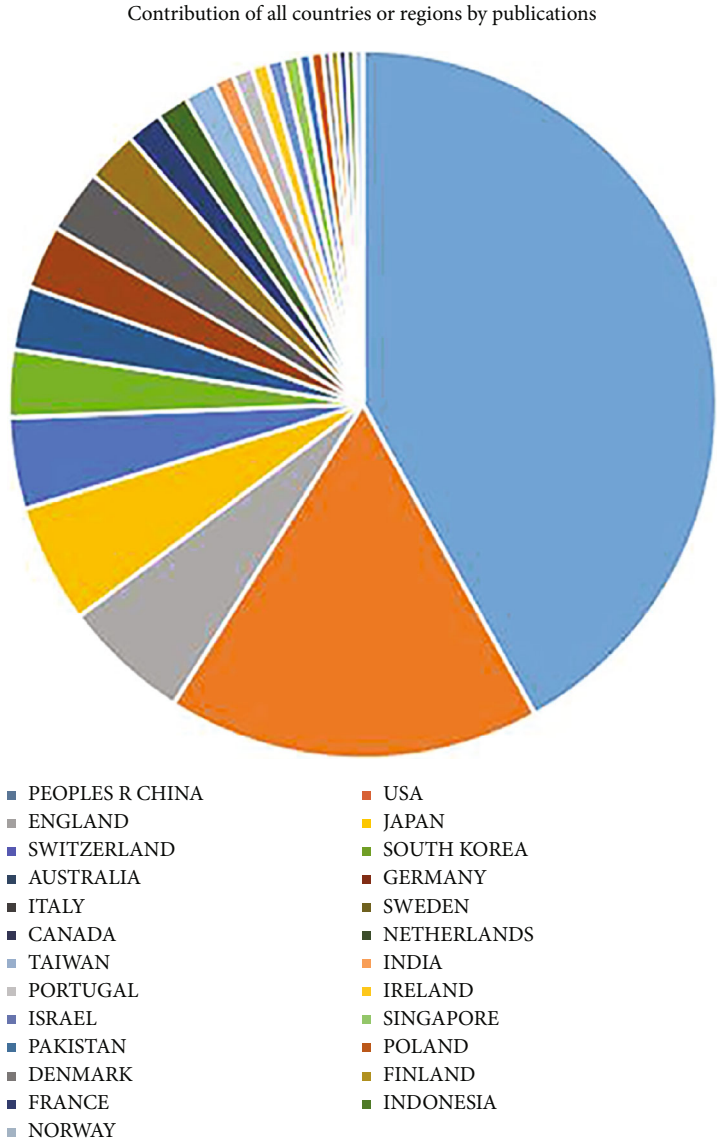
Figure 1: Annual publications and sum of times cited per year on stem and progenitor cells in intervertebral discs.

University, Army Medical University, League of European Research Universities-LERU, Huazhong University of Science & Technology, and University of Hong Kong had contributed 33, 29, 29, 26, and 18 publications, respectively. The top 5 countries with the most publications were China ($n = 231$), the USA ($n = 94$), England ($n = 32$), Japan ($n = 30$), and Switzerland ($n = 23$). What's more, the corresponding records, H-index, total citations of the top 5 authors, organizations, and countries were meanwhile showed in Table 2.

3.5. Contribution of Research Directions, Funds, and Journals. There were 38 research directions, 478 funds, and 167 Journals contributed to publications on stem and progenitor cells in intervertebral discs, respectively. Cell biology occupied the most records ($n = 181$), the highest H-index of 35, the highest total citations ($n = 4421$), and average citations ($n = 24.43$) [19]. Neurosciences and neurology occupied the most average citations ($n = 52.94$). Orthopedics had the second records ($n = 92$), the highest H-index of 35, the second total citations ($n = 4347$), and the second average citations ($n = 47.25$) [20]. In addition, National Natural Science Foundation of China had the most records ($n = 157$), the highest H-index of 27, the highest total citations ($n = 2798$), and average citations ($n = 17.82$) [21, 22]. National Institutes of Health (NIH), USA, and United States Department of Health & Human Services were the second with records ($n = 30$), H-index of 19, the total citations ($n = 1239$), and the highest average citations ($n = 41.30$). Furthermore, *Spine* occupied the most records ($n = 27$), the highest H-index of 20, the highest total citations ($n = 2025$), and the highest average citations ($n = 75.00$) [23, 24]. *Stem Cells International* was the second with records ($n = 23$), H-index of 12, the total citations ($n = 359$), and average citations ($n = 15.61$) [25–27]. Furthermore, the corresponding records, H-index, total citations, and average citations of the top 5 research directions, funds, and journals with the most publications were meanwhile list in Table 3.

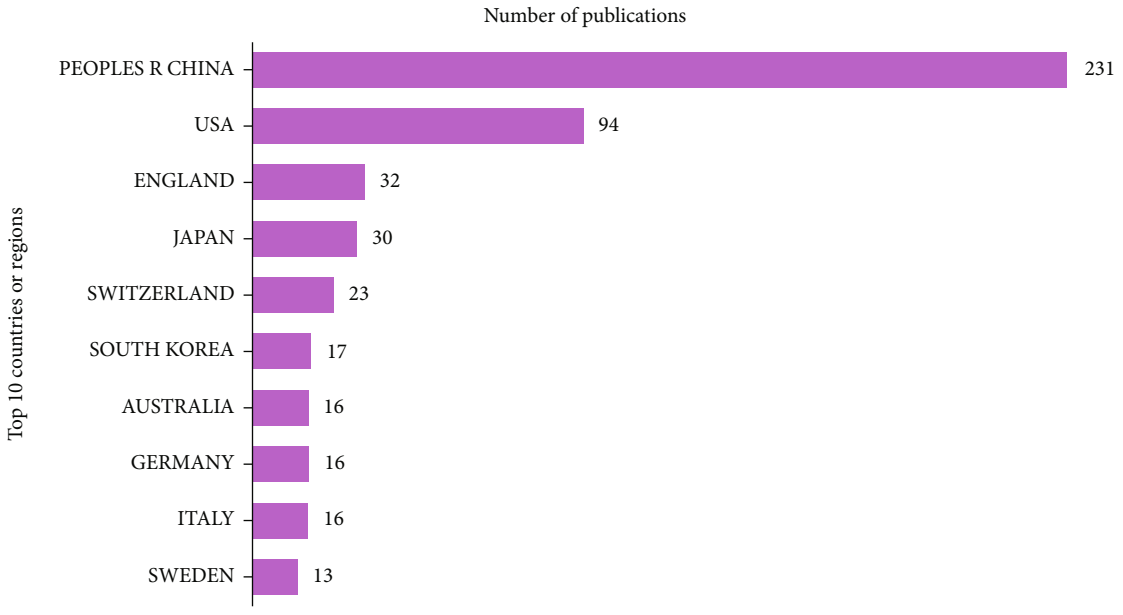
3.6. Co-Authorship Analysis of Publications. Zhou XP had the most co-authorship strength (total link strength = 87), with 17 documents and 348 citations [28], followed by Liang CZ (total link strength = 85) with 16 documents and 392 citations [29] and Li FC (total link strength = 84) with 16 documents and 358 citations [30] (Figure 3(a)). Moreover, the closest collaboration organization was Shanghai Jiao Tong University (total link strength = 20) with 15 documents and 290 citations, the second organization was Yangzhou University (total link strength = 18) with 13 documents and 224 citations, and the third was Chinese Orthopaedic Regenerative Medicine Society (total link strength = 14) with 6 documents and 239 citations [31–33] (Figure 3(b)). Besides, the strongest collaborative country was the USA (total link strength = 58) with 94 documents and 4791 citations, followed by China (total link strength = 35) with 231 documents and 4522 citations and Japan (total link strength = 24) with 30 documents and 2612 citations (Figure 3(c)).

3.7. Co-Citation Analysis of Publications. The most co-citation reference ($n = 96$) titled "Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy" was published by Risbud MV on *Spine* in 2004 [34]. The second reference ($n = 92$) titled "Differentiation of mesenchymal stem cells transplanted to a rabbit degenerative disc model - Potential and limitations for stem cell therapy in disc regeneration" was published by Sakai D on *Spine* in 2005 [35], The third reference ($n = 92$) was "Transplantation of mesenchymal stem cells embedded in Atelocollagen((R)) gel to the intervertebral disc: a potential therapeutic model for disc degeneration", published by Sakai D on *Biomaterials* in 2003 (Figure 4(a)). On the other hand, the most co-citation journal was *Spine* ($n = 3179$) [36], followed by *European Spine Journal* ($n = 774$) [37] and *Spine Journal* ($n = 582$) [38] (Figure 4(b)). Furthermore, the most co-citation author was Sakai D ($n = 547$) [9], the second

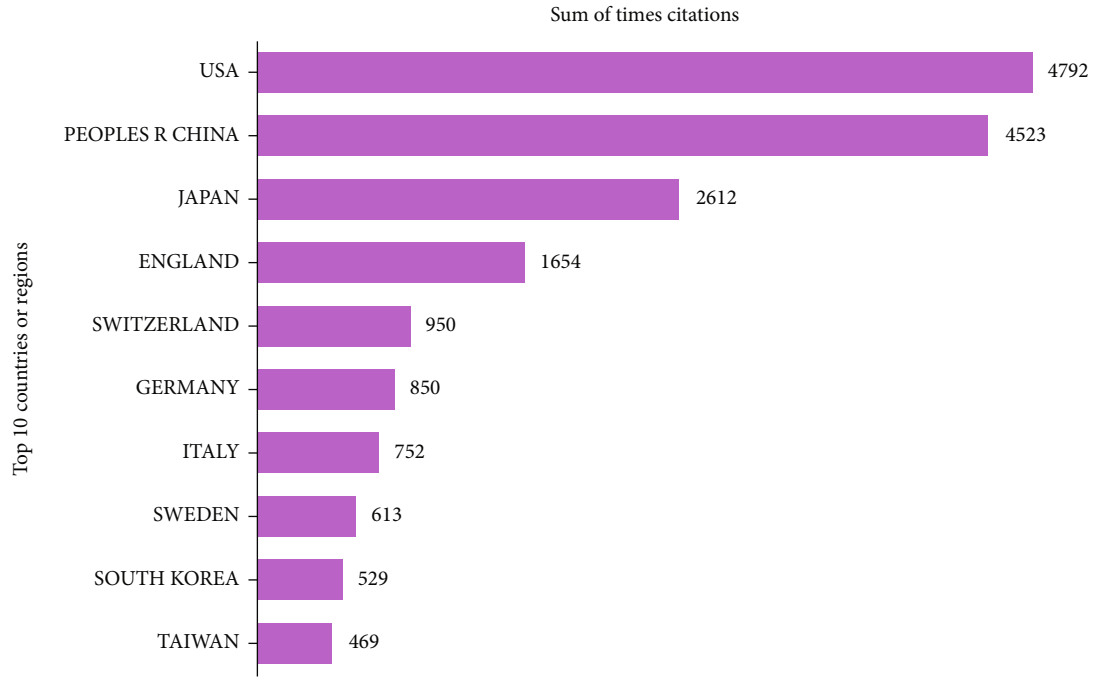


(a)

Figure 2: Continued.



(b)



(c)

Figure 2: Continued.

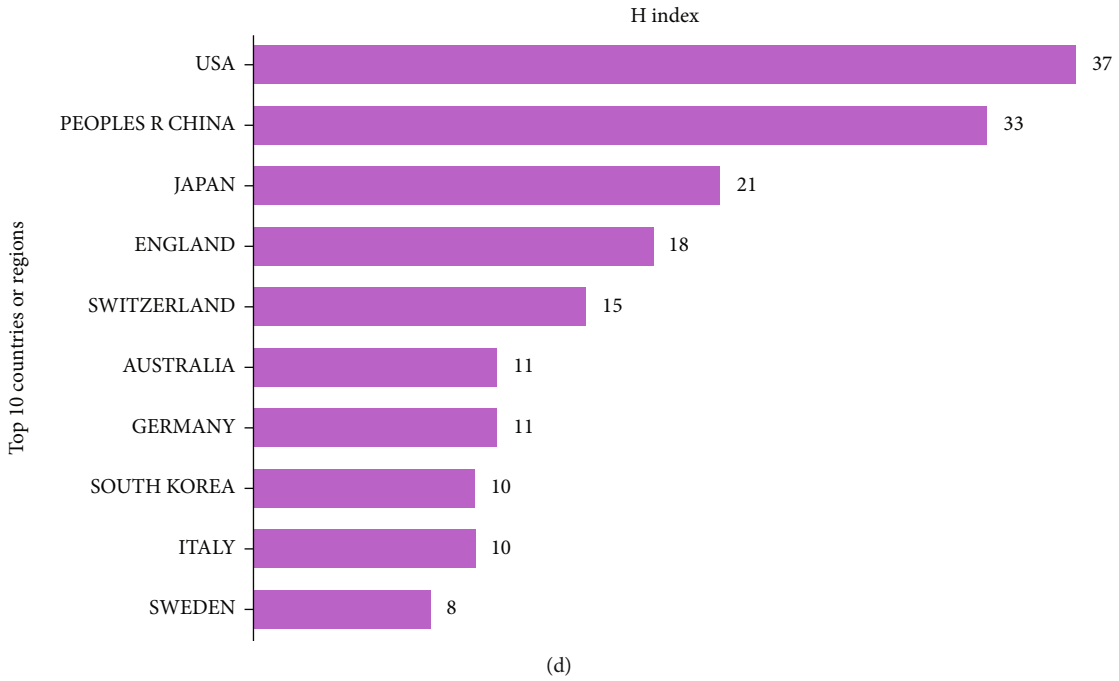


Figure 2: (a) Contribution of all countries by Publications. (b–d) Total number of publications, sum of total citations, and H-index of top 10 countries on stem and progenitor cells in intervertebral discs.

was Risbud MV ($n = 333$) [34], and the third was Richardson SM ($n = 234$) [39] (Figure 4(c)).

3.8. Co-Citation Timeline of References and Burst Analysis. Co-citation of references for a timeline diagram was drawn by Citespace software (Figure 5). References to the same cluster are arranged on the timeline in chronological order of publication. "bilaminar pellet", "apoptosis", and "phenotypic markers" were the clusters with most published references. According to the year of publication, "tissue engineering", "angiogenesis", and "tgf-beta" were the clusters with the earliest references. "Apoptosis" and "phenotypic markers" were the clusters with the latest references. The top 25 references with the highest burst value are shown in Figure 6. The earliest reference with the strongest citation bursts was "Transplantation of mesenchymal stem cells embedded in Atelocollagen((R)) gel to the intervertebral disc: a potential therapeutic model for disc degeneration", published by Sakai D on *Biomaterials* in 2003 [39]. The latest reference with the strongest citation bursts was "Mesenchymal stem cells deliver exogenous miR-21 via exosomes to inhibit nucleus pulposus cell apoptosis and reduce intervertebral disc degeneration", published by Cheng XF on *Journal of Cellular and Molecular Medicine* in 2018 [18].

3.9. Keyword Visualization Analysis. The log-likelihood rate (LLR) algorithm was used to cluster all keywords by Citespace software, and the top 9 clusters are shown in Figure 7 and Table 4. Generally speaking, clustering module value $(Q) > 0.3$, indicating that the clustering structure is significant; the average contour value $(S) > 0.7$ means that the clustering is convincing. $Q = 0.4569$, and $S = 0.7188$ in our study. Each label was interconnected and developed, not independently exist. The color corresponding to the cluster area indicated the first time that a co-citation appeared. The clusters represented by green appeared later than the clusters represented by blue and purple. The smaller the cluster number stood for the more keywords the cluster contained. The cluster labels were as follows: #0 human intervertebral disc, #1 adipose-derived mesenchymal stem cell, #2 intervertebral disc degeneration, #3 degenerative disc model, #4 nucleus pulposus regeneration, #5 human cartilage, #6 3d culture, #7 shrinkage-free preparation, and #8 polylactide disc.

The top 14 keywords with the highest burst value are illustrated in Figure 8. The time period occupied by red on the right was the duration of the keywords. According to the burst strength and duration of the keywords, the transformation of domain research direction can be roughly divided into three stages. The first stage was from 2003 to 2009; the keywords were "in vivo", "disc degeneration", "disc regeneration", and "bone marrow". The second stage was from 2010 to 2016; the keywords were "tissue", "chondrogenesis", "growth", and "proliferation". The third stage was from 2017 to 2021; the keywords were "microenvironment", "activation", "intervertebral disc degeneration", and "oxidative stress".

4. Discussion

4.1. General Information and Bibliometric Analysis. The number of publications on a specific topic can reflect the popularity in this field. The researches regarding on stem and progenitor cells in intervertebral discs was initially published in 1999. The number of articles published increased rapidly from 2002 to 2022. On the other hand, the quality

Table 1: Top 20 most cited articles on stem and progenitor cells in intervertebral discs.

First author	Article title	Journal	Publication year	Total citations	Impact factor
Sakai, D	Transplantation of mesenchymal stem cells embedded in Atelocollagen((R)) gel to the intervertebral disc: a potential therapeutic model for disc degeneration	Biomaterials	2003	311	14.593
Sakai, D	Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc	Biomaterials	2006	277	14.593
Sakai, D	Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc	Nature Communications	2012	267	14.919
Sakai, D	Differentiation of mesenchymal stem cells transplanted to a rabbit degenerative disc model - potential and limitations for stem cell therapy in disc regeneration	Spine	2005	265	3.468
Richardson, SM	Mesenchymal stem cells in regenerative medicine: docus on articular cartilage and intervertebral disc regeneration	Methods	2016	251	3.608
Risbud, MV	Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy	Spine	2004	250	3.468
Crevensten, G	Intervertebral disc cell therapy for regeneration: mesenchymal stem cell implantation in rat intervertebral discs	Annals of Biomedical Engineering	2004	241	3.934
Sakai, D	Stem cell therapy for intervertebral disc regeneration: obstacles and solutions	Nature Reviews Rheumatology	2015	240	20.543
Richardson, SM	Intervertebral disc cell-mediated mesenchymal stem cell differentiation	Stem Cells	2006	227	6.277
Risbud, MV	Evidence for skeletal progenitor cells in the degenerate human intervertebral disc	Spine	2007	213	3.468
Steck, E	Induction of intervertebral disc-like cells from adult mesenchymal stem cells	Stem Cells	2005	202	6.277
Dang, JM	Temperature-responsive hydroxybutyl chitosan for the culture of mesenchymal stem cells and intervertebral disk cells	Biomaterials	2006	190	14.593
Vadala, G	Mesenchymal stem cells injection in degenerated intervertebral disc: cell leakage may induce osteophyte formation	Journal of Tissue Engineering and Regenerative Medicine	2012	187	3.963
Hiyama, A	Transplantation of mesenchymal stem cells in a canine disc degeneration model	Journal of Orthopaedic Research	2008	184	2.359
Minogue, BM	Characterization of the human nucleus pulposus cell phenotype and evaluation of novel marker gene expression to define adult stem cell differentiation	Arthritis and Rheumatism	2010	161	8.955
Arai, F	Mesenchymal stem cells in perichondrium express activated leukocyte cell adhesion molecule and participate in bone marrow formation	Journal of Experimental Medicine	2002	158	14.307
Henriksson, HB	Transplantation of human mesenchymal stems cells into intervertebral discs in a xenogeneic porcine model	Spine	2009	151	3.468
Henriksson, HB	Identification of cell proliferation zones, progenitor cells and a potential stem cell niche in the intervertebral disc region A study in four species	Spine	2009	148	3.468
Richardson, SM	Mesenchymal stem cells in regenerative medicine: opportunities and challenges for articular cartilage and intervertebral disc tissue engineering	Journal of Cellular Physiology	2010	143	6.384
Sobajima, S	Feasibility of a stem cell therapy for intervertebral disc degeneration	Spine Journal	2008	141	4.166

Table 2: The top 5 high-yield authors, organizations, and countries on stem and progenitor cells in intervertebral discs.

Category	Rank	Items	Records	H-index	Total citations	Average citations
Author	1	Chen, QX, Zhejiang University	22	15	571	25.95
	1	Zhou, Y, Army Medical University	22	12	544	24.73
	3	Li, FC, Zhejiang University	20	15	527	26.35
	3	Liang, CZ, Zhejiang University	20	15	560	28.00
	5	Li, H, Shanghai Jiao Tong University	19	15	637	33.53
Organization	1	Zhejiang University	33	16	726	22.00
	2	Army Medical University	29	13	609	21.00
	2	League of European Research Universities-LERU	29	18	1382	47.66
	4	Huazhong University of Science & Technology	26	13	419	16.12
	5	University of Hong Kong	18	15	1092	60.67
Country	1	China	231	33	4523	19.58
	2	USA	94	37	4792	50.98
	3	England	32	18	1654	51.69
	4	Japan	30	21	2612	87.07
	5	Switzerland	23	15	950	41.30

Table 3: The top 5 high-yield research directions, funds, and journals with the most publications on stem and progenitor cells in intervertebral discs.

Category	Rank	Items	Records	H-index	Total citations	Average citations
Research direction	1	Cell Biology	181	35	4421	24.43
	2	Orthopedics	92	35	4347	47.25
	3	Research & Experimental Medicine	78	24	1997	25.60
	4	Engineering	74	28	3034	41.00
	5	Neurosciences & Neurology	63	33	3335	52.94
Fund	1	National Natural Science Foundation of China (NSFC)	157	27	2798	17.82
	2	National Institutes of Health (NIH) - USA	30	19	1239	41.30
	2	United States Department of Health & Human Services	30	19	1239	41.30
	4	European Commission	18	11	545	30.28
	4	National Key Research and Development Program of China	18	11	345	19.17
Journal	1	Spine	27	20	2025	75.00
	2	Stem Cells International	23	12	359	15.61
	3	Tissue Engineering Part A	16	13	611	38.19
	4	Spine Journal	15	12	669	44.60
	5	Stem Cell Research & Therapy	13	9	348	26.77

on a specific topic can be judged by the number of citations. There was a linear growth for the citation times from 1999 to 2022. From the result of Figure 1, we can know that the future trend on stem and progenitor cells in intervertebral discs looks very promising.

China was dominant in this field by the number of publications. Meanwhile, the publications of China and the USA accounted for 72.22%, which indicates a great contribution to this field by these two countries. It was might associated with large disc degeneration populations and high incidence in two countries [40]. China had the most publications. However, the USA had the highest citations and H-index, showing that China's research in this field was not deep enough. Sakai D, coming from Tokai University School of Medicine in Japan, has 5 most cited articles in the top 20 and 4 articles among them listed in top 4, with more than 265 total citations. He focused on mesenchymal stem cells embedded in Atelocollagen((R)) gel to the intervertebral disc and stem cell therapy for intervertebral disc regeneration [41].

From the analysis of number of publications issued by the author, the top 5 authors all came from China. Professor

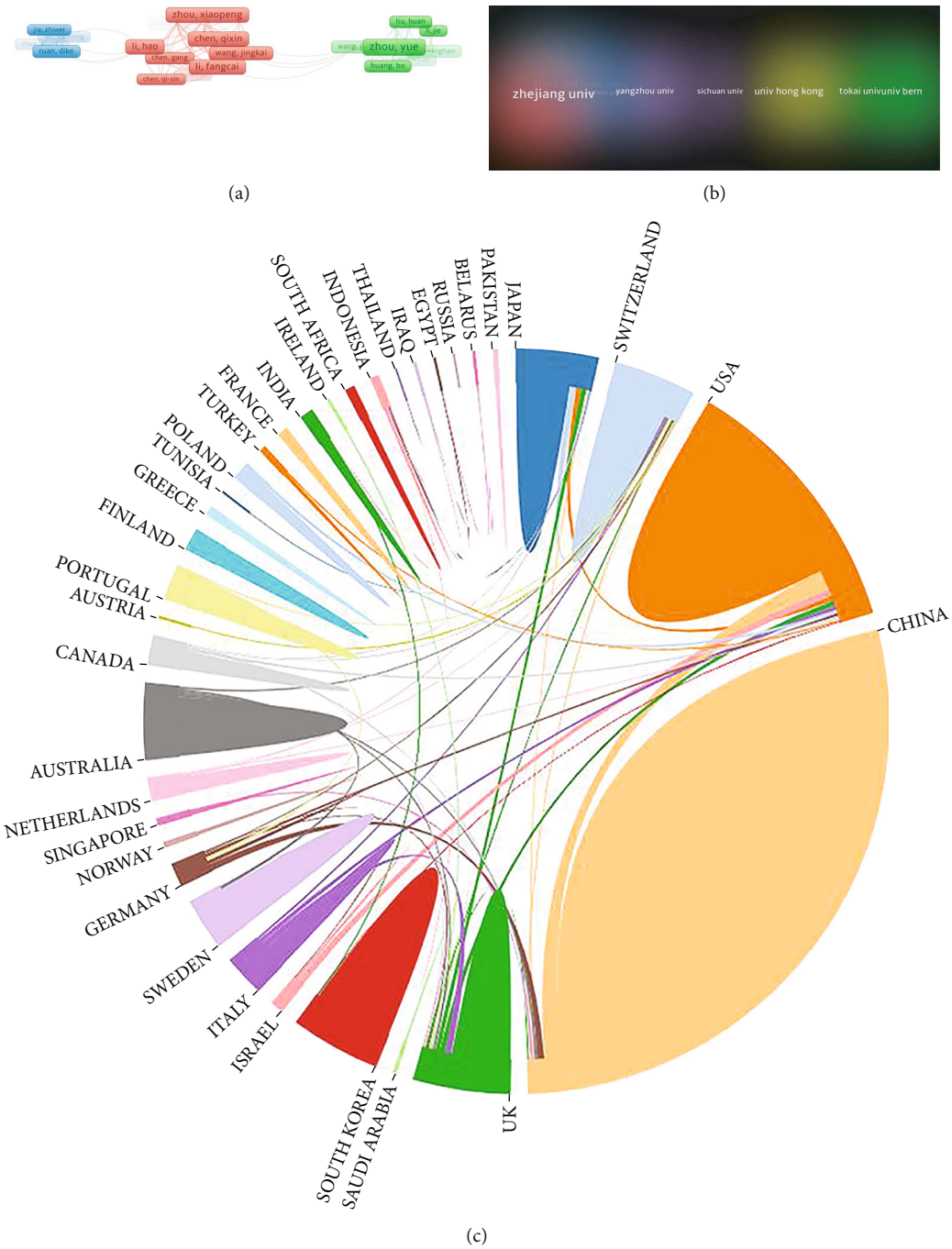
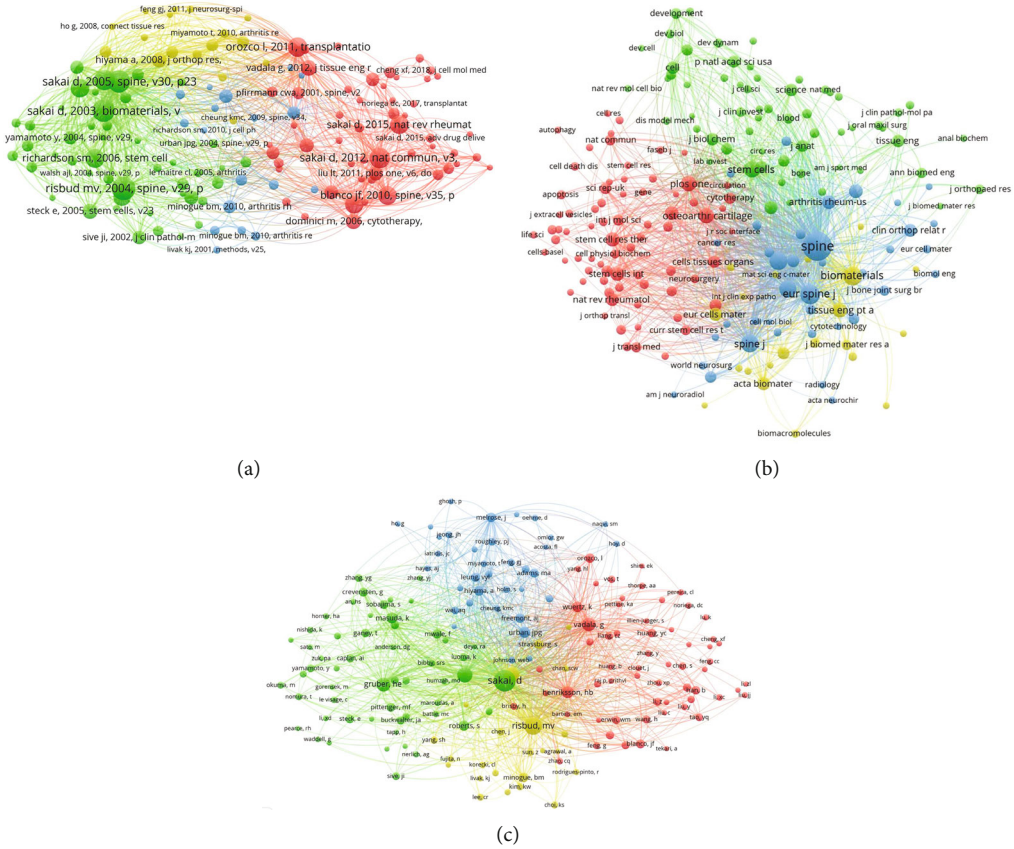


Figure 3: The co-authorship analysis of (a) authors, (b) organizations, and (c) countries on stem and progenitor cells in intervertebral discs. (The size of the frames represents the proportion of the author in the analysis. The larger the frames, the greater the contribution. The line between the frames represents the connection between the authors. The more or thicker the line, the stronger the connection. The color of the area where organization is located represents the connection between organizations. The darker the color, the closer the collaboration organization; the larger the area, the greater the contribution).

Chen QX from Zhejiang University in China has a great influence in this field. They had carried out numbers of researches on improving the biological repair function of nucleus pulposus mesenchymal stem cells by constructing biological scaffolds and introducing growth factors [42, 43]. Professor Zhou Yue from the Chinese Army Military Medical University was also made great contributions to the research direction of mesenchymal stem cell differentiation [42, 43].

Zhejiang University and Army Medical University were listed in the top 2, and only one institution was not in China in the top 5, which demonstrated that Chinese universities



(a) (b)

(c)

Figure 4: The co-citation analysis of (a) references, (b) journals, and (c) authors on stem and progenitor cells in intervertebral discs. (A point in the figure represents one reference, journal, and author, respectively. The color of the point represents different clusters; the size of the point represents the number of citations for each reference, journal, and author, respectively. The more the number, the larger the point. The connection between the two points represents two papers are jointly cited by another paper, and the length of the connection between the two points represents the correlation between two articles; the shorter the line, the stronger the correlation).

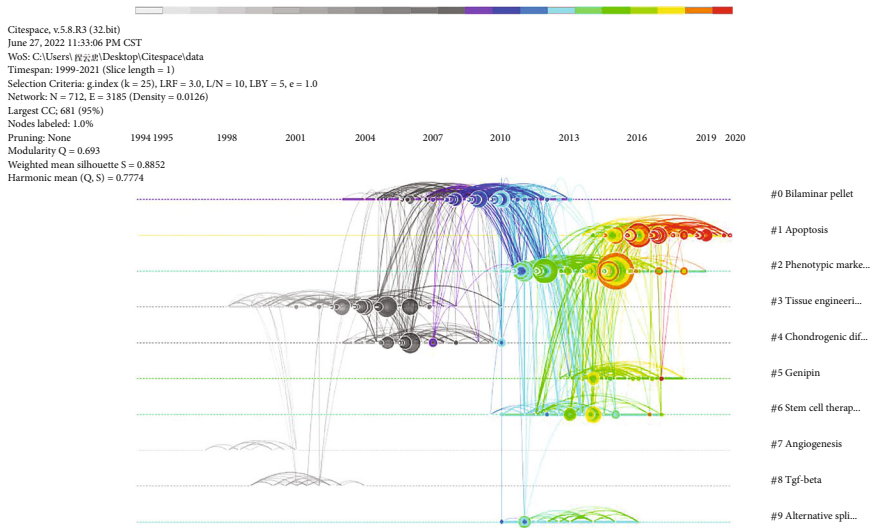


Figure 5: Co-citation timeline of references by keywords on stem and progenitor cells in intervertebral discs. (The nodes represent the references. The larger the node, the more citations the reference. The colors of the nodes from the inside to the outside correspond to color scale, which represents the total co-citations for the reference in the specific year. The line between two nodes represents two references co-citations. The thicker the line, the more the co-citations. The color of the connection line corresponds to the color mark above, which can reflect the time when two references were first co-cited.)

Top 25 references with the strongest citation bursts

References	Year	Strength	Begin	End	1999-2021
Sakai D, 2003, BIOMATERIALS, V24, P3531, DOI 10.1016/S0142-9612 (03) 00222-9, DOI	2003	9.27	2004	2008	
Crevensten G, 2004, ANN BIOMED ENG, V32, P430, DOI 10.1023/B: ABME.0000017545.84833.7c, DOI	2004	8.61	2005	2009	
Sakai D, 2006, BIOMATERIALS, V27, P335, DOI 10.1016/j.biomaterials.2005.06.038, DOI	2006	12.23	2006	2011	
Steck E, 2005, STEM CELLS, V23, P403, DOI 10.1634/stemcells.2004-0.107, DOI	2005	10.73	2006	2010	
Risbud MV, 2004, SPINE, V29, P2627, DOI 10.1097/01.brs.0000146462.92171.7f, DOI	2004	9.4	2006	2009	
Yamamoto Y, 2004, SPINE, V29, P1508, DOI 10.1097/01.BRS.0000131416.90906.20, DOI	2004	6.61	2006	2009	
Zhang YG, 2005, CLIN ORTHOP RELAT R, V0, P0, DOI 10.1097/01.b1o.0000146534.31120.cf, DOI	2005	6.61	2006	2010	
Sakai D, 2005, SPINE, V30, P2379, DOI 10.1097/01.brs.0000184365.28481.e3, DOI	2005	12.32	2007	2010	
Richardson SM, 2006, STEM CELLS, V24, P707, DOI 10.1634/stemcells.2005-0205, DOI	2006	10.86	2007	2011	
Sobajima S, 2008, SPINE J, V8, P888, DOI 10.1016/j.spinee.2007.09.011, DOI	2008	6.72	2010	2013	
Henriksson HB, 2009, SPINE, V34, P141, DOI 10.1097/BRS.0b013e31818f8c20, DOI	2009	6.68	2010	2014	
Blanco JF, 2010, SPINE, V35, P2259, DOI 10.1097/BRS.0b013e3181cb8828, DOI	2010	8.29	2011	2015	
Strassburg S, 2010, REGEN MED, V5, P701, DOI 10.2217/RME.10.59, DOI	2010	7.45	2011	2015	
Stoyanov JV, 2011, EUR CELLS MATER, V21, P533, DOI 10.22203/eCM.v021a40, DOI	2011	9.62	2012	2016	
Orozco L, 2011, TRANSPLANTATION, V92, P822, DOI 10.1097/TP.0b013e3182298a15, DOI	2011	7.7	2013	2016	
Vadala G, 2012, J TISSUE ENG REGEN M, V6, P348, DOI 10.1002/term.433, DOI	2012	7.58	2013	2017	
Liu LT, 2011, PLOS ONE, V6, P0, DOI 10.1371/journal.pone.0026285, DOI	2011	6.71	2014	2016	
Sakai D, 2012, NAT COMMUN, V3, P0, DOI 10.1038/ncomms2226, DOI	2012	9.9	2015	2017	
Huang YC, 2013, SPINE J, V13, P352, DOI 10.1016/j.spinee.2012.12.005, DOI	2013	7	2015	2018	
Sakai D, 2015, NAT REV RHEUMATOL, V11, P243, DOI 10.1038/nrrheum.2015.13, DOI	2015	13.87	2016	2021	
Han B, 2014, CELLS TISSUES ORGANS, V199, P342, DOI 10.1159/000369452, DOI	2014	6.44	2016	2019	
Richardson SM, 2016, METHODS, V99, P69, DOI 10.1016/j.ymeth.2015.09.015, DOI	2016	7.16	2018	2021	
Liu JJ, 2017, STEM CELLS DEV, V26, P901, DOI 10.1089/scd.2016.0314, DOI	2017	6.65	2018	2021	
Cheng XF, 2018, J CELL MOL MED, V22, P261, DOI 10.1111/jcmm.13316, DOI	2018	7.13	2019	2021	
Lu K, 2017, STEM CELL RES THER, V8, P0, DOI 10.1186/s13287-017-0563-9, DOI	2017	6.68	2019	2021	

Figure 6: Top 25 references with the strongest citation bursts on stem and progenitor cells in intervertebral discs.

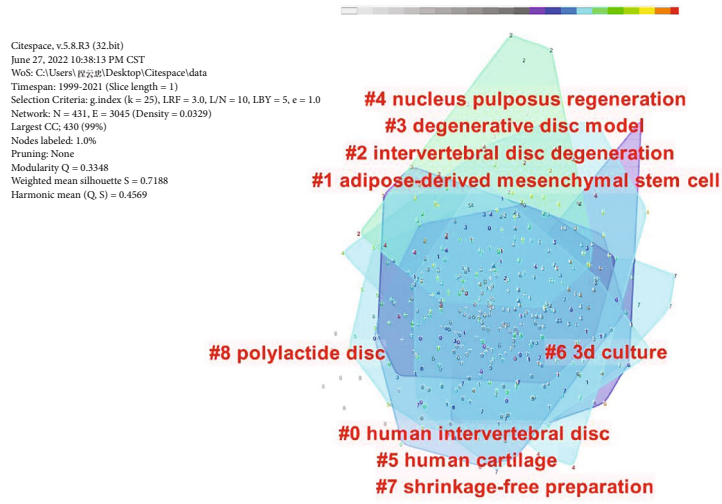


Figure 7: Keywords clusters on stem and progenitor cells in intervertebral discs. (A point in the figure represents a keyword. The color of the point represents different clusters, and the size of the point represents the co-occurrence for each keyword. The more the co-occurrence, the larger the point; # represents different cluster labels, #0 human intervertebral disc, #1 adipose-derived mesenchymal stem cell, #2 intervertebral disc degeneration, #3 degenerative disc model, #4 nucleus pulposus regeneration, #5 human cartilage, #6 3d culture, #7 shrinkage-free preparation, and #8 polylactide disc.)

and institutions had a great contribution and influence in the field of stem and progenitor cells in intervertebral discs. From the analysis of number of publications issued by fund and journal, National Natural Science Foundation of China (NSFC) and National Institutes of Health (NIH), USA, were in the top 2, which was consistent with the greatest contribution of China and the USA. *Spine* and *Stem Cells International* were listed top 2 in all journals. The co-authorship analysis of authors, organizations, and countries on stem and progenitor cells in intervertebral discs showed the cooperation between them was not closely enough.

4.2. Research Hotspots on Stem and Progenitor Cells in Intervertebral Discs. Through the cluster analysis of keywords, we can clearly know the research hotspots on stem and progenitor cells in intervertebral discs.

Table 4: Details of top 9 clusters for researches on stem and progenitor cells in intervertebral discs.

Cluster no.	Size (n)	Silhouette	Mean (year)	LSI	LLR	MI
0	65	0.605	2011	Mesenchymal stem cell; intervertebral disc; stem cell; intervertebral disc degeneration; nucleus pulposus | nucleus pulposus cell; disc cell; mesenchymal stem; disc degeneration; disc regeneration	Human intervertebral disc; skeletal progenitor cell; human cartilage endplate; human nucleus; potential stem cell niches	Phenotypic marker; human degenerative intervertebral disc; iron oxide; cell survival; hypoxic-preconditioned bone
1	64	0.665	2012	Mesenchymal stem cell; adipose-derived stem cell; nucleus pulposus; nucleus pulposus cell; intervertebral disc | marrow-derived mesenchymal stem cell; rabbit model; signaling pathway; pulposus-like differentiation; collagen-induced nucleus	Adipose-derived mesenchymal stem cell; adipose-derived stem cell; co-culture system; nucleus pulposus; pulposus-like cell	Phenotypic marker; human degenerative intervertebral disc; iron oxide; cell survival; hypoxic-preconditioned bone
2	56	0.817	2011	Mesenchymal stem cell; intervertebral disc degeneration; nucleus pulposus cell; intervertebral disc; stem cell | nucleus pulposus; human cartilage; progenitor cell; adipose-derived stem cell; pulposus-derived mesenchymal stem cell	Intervertebral disc degeneration; nutrition deficiency; autologous hematopoietic progenitor cell support; high-dose chemotherapy; optic disc	Phenotypic marker; human degenerative intervertebral disc; iron oxide; cell survival; hypoxic-preconditioned bone
3	52	0.747	2009	Mesenchymal stem cell; intervertebral disc; nucleus pulposus cell; human mesenchymal stem cell; degenerative disc model | intervertebral disc degeneration; nucleus pulposus; rabbit model; bone marrow; adipose-derived stem cell	Degenerative disc model; disc regeneration; canine disc degeneration model; disc cell; AKT axis	Phenotypic marker; human degenerative intervertebral disc; iron oxide; cell survival; hypoxic-preconditioned bone
4	49	0.718	2016	Mesenchymal stem cell; intervertebral disc; nucleus pulposus; stem cell; intervertebral disc degeneration | progenitor cell; regenerative medicine; articular cartilage; enhanced regenerative effect; adipose stem	Nucleus pulposus regeneration; annulus fibrosus regeneration; pulposus-based cell delivery system; cyclic compression; perfusion bioreactor	Cell survival; hypoxic-preconditioned bone; phenotypic marker; human degenerative intervertebral disc; iron oxide
5	43	0.735	2014	Mesenchymal stem cell; intervertebral disc; human cartilage; endplate-derived stem cell; splicing event | nucleus pulposus progenitor cell; mesenchymal stem cell differentiation; disc cell; collagen type ii; hypoxic condition	Human cartilage; endplate-derived stem cell; splicing event; genome-wide analysis; stromal cell	Human degenerative intervertebral disc; phenotypic marker; iron oxide; cell survival; hypoxic-preconditioned bone
6	37	0.725	2011	Mesenchymal stem cell; nucleus pulposus cell; human mesenchymal stem cell; vitro study; pulposus-like cell | adipose stem cell; configuration effect; co-cultured stem cell; matrix production; low back pain patient	3d culture; alginate beads hypoxia bone; synthetic peptide b2a; modeling nucleus; vitro study	Phenotypic marker; human degenerative intervertebral disc; iron oxide; cell survival; hypoxic-preconditioned bone
7	37	0.681	2013	Mesenchymal stem cell; nucleus pulposus; scaffold-free cartilage-like disc-shaped cell sheet; shrinkage-free preparation; using human bone marrow | intervertebral disc degeneration; human mesenchymal stem cell; pentosan polysulfate; ovine model; mesenchymal progenitor cell	Shrinkage-free preparation; scaffold-free cartilage-like disc-shaped cell sheet; using human bone marrow; human placenta-derived mesenchymal stem cell; functional regeneration	Phenotypic marker; human degenerative intervertebral disc; iron oxide; cell survival; hypoxic-preconditioned bone
8	27	0.887	2005	Polylactide disc; temporomandibular joint disc; mesenchymal stem cell; intervertebral disc; tissue engineering | nucleus pulposus; bone marrow stem cell; differential response; chitosan hydrogel; iron oxide	Polylactide disc; temporomandibular joint disc; bone marrow formation; tissue engineering; leukocyte cell adhesion molecule	Mesenchymal stem cell; intervertebral disc; nucleus pulposus cell; iron oxide; phenotypic marker

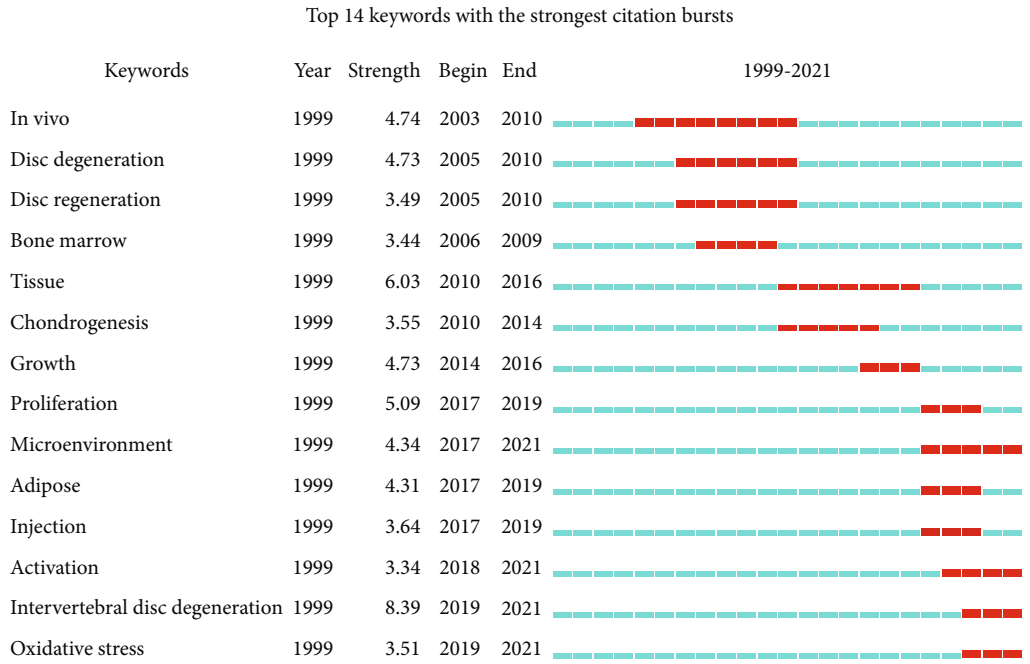


Figure 8: Top 14 keywords with the strongest citation bursts on stem and progenitor cells in intervertebral discs.

4.2.1. Cluster #0 Human Intervertebral Disc. The intervertebral disc is located between adjacent vertebral bodies and consists of the peripheral annulus fibrosus (annulus fibrosus, AF), central jelly-like nucleus pulposus (nucleus pulposus, NP), and cartilage endplate (cartilage endplate, CE). The peripheral AF is mainly composed of fibroblast-like annulus fibrosus. And the main cell types of NP are stellate chondroid cells. The bone endplate is a thin layer of cartilage, which is similar with articular cartilage tissue [44]. As all we know, human intervertebral discs include NP, AF, hyaline CE, and disc perichondrium. At present, stem cell therapy mainly focuses on NP and AF [45–48].

4.2.2. Cluster #1 Adipose-Derived Mesenchymal Stem Cell, ADMSC. ADMSCs are stem cells with pluripotent differentiation potential, which isolated from adipose tissue that is widely and readily available nowadays. It is the current research hotspot of stem cell therapy for degenerative disc diseases (DDD). Studies have shown that compared with bone marrow derived mesenchymal stem cell (BMSCs), ADMSCs have a higher nucleus pulposus-like differentiation capacity. Therefore, ADMSC may be more suitable for the treatment of DDD [49].

Clarke et al. found that the ability of ADMSC to differentiate into nucleus pulposus cell phenotype was strongly enhanced under the induction of TGF-β1, GDF5, and GDF6. The cell culture medium level of sulfated glycosaminoglycans and COL II significantly increased [50].

Han et al. performed the analysis of ADMSCs and degenerated nucleus pulposus cells. In vitro co-culture, it not only demonstrated that ADMSCs could promote the repair of degenerated nucleus pulposus cells, but also the first comprehensive identification of degenerative myeloid when co-cultured with ADMSCs nuclear cells were capable of producing lncRNA and mRNA differentially expressed [51]. These research results further provided more valuable information so that people can better understand the role of stem cell therapy in IDD.

The current study shows that ADMSC can be successfully induced to differentiate into nucleus pulposus-like cells under certain conditions to repair the degenerated intervertebral disc, and partially grow biofactors can enhance the repair of ADMSCs. However, the long-term efficacy and safety of ADMSC clinical trials still need to be further verified [52].

4.2.3. Cluster #2 Intervertebral Disc Degeneration, IDD. There are many factors for the degeneration of intervertebral disc, but the most important reason is the decrease of apoptosis and activity of nucleus pulposus cells, which is recognized by the world. The nucleus pulposus cells in the intervertebral disc are in a microenvironment such as hypoxia, acidity, hypertonicity, and lack peripheral blood nutrient supply, which are totally different from other cells in the intervertebral disc [53].

However, the number of NP cells in the intervertebral disc is small, and the rate of cell regeneration is lower than the rate of apoptosis and aging. Under natural conditions, it is difficult for the degenerated intervertebral disc to regenerate and repair to achieve the desired effect. Therefore, it is urgent to find a seed cell that can replace the degenerated NP cells to delay the process of intervertebral disc degeneration. NP cells and chondrocytes are very similar in terms of molecular markers and cell phenotypes, so stem cells that

can differentiate into chondrocytes are the best source of cell transplantation for the treatment of intervertebral disc degeneration [54].

Therefore, IDD is difficult to repair by itself and is irreversible. In view of this feature, more and more teams have begun to use stem cells to intervene in the IDD process in order to slow down the IDD process or repair the degenerated intervertebral disc, including mesenchymal stem cells (MSC), intervertebral disc-derived stem cells (IVDSC), and pluripotent stem cells (PSC) [55].

4.2.4. Cluster #3 Degenerative Disc Model. At present, there are more than a dozen animals used to construct intervertebral disc degeneration models, such as mice, rabbits, dogs, pigs, sheep, cattle, and primates. Primates such as monkeys and orangutans are close relatives of humans, and their intervertebral discs are quite similar with human intervertebral discs in terms of physiological structure and biomechanics. However, the current animal experimental research is limited by animal sources, experimental funds, practical operations, and ethics. Therefor primates are rarely used to construct intervertebral disc degeneration models. In addition, large mammals also have disadvantages such as high price and difficulty in feeding [56].

Nowadays, the ideal animal models are small animals such as rabbits and rats. These two animals have the advantages of pure species, many sources, easy to raise, and low price. Although the intervertebral discs of tetrapods lack comparison with human intervertebral discs, they have made great contributions to the pathogenesis and treatment of intervertebral discs [57].

4.3. Research Frontiers and Trends on Stem and Progenitor Cells in Intervertebral Discs. The analysis of keyword bursts can grasp the research frontier and latest progress in the field of stem and progenitor cells in intervertebral discs. 4 keywords with the strongest citation bursts appeared from 2017 to 2021, including "microenvironment", "activation", "intervertebral disc degeneration", and "oxidative stress".

In recent years, related studies have shown that intervertebral disc degeneration is not only affected by the environment and genes, but also related to the microenvironment in intervertebral disc, such as oxygen content, nutrients, and growth factors, which can deteriorate the metabolic environment of nucleus pulposus cells, strengthen anaerobic metabolism, accumulate lactic acid, change acidity, and aggravate intervertebral disc degeneration [58]. Bibby et al. studied the standard unit of lumbar intervertebral disc and found that the glucose concentration and oxygen partial pressure in the endplate area were positively correlated with the cell density in the nucleus pulposus and inversely proportional to the lactate concentration [59], while Mokhbi Soukane studied the standard unit of lumbar intervertebral disc. It was confirmed that the lactate concentration in the blood supply of the endplate cartilage was positively correlated with the degeneration of the intervertebral disc in the corresponding stage [60].

According to the free radical theory of aging, the decline of tissue and organ function is closely related to the oxidative stress induced by reactive oxygen species (ROS) [61]. The occurrence and progression of intervertebral disc degeneration is no exception [62, 63]. In the signaling pathway network of nucleus pulposus cells, ROS acts as an important mediator, regulating extracellular matrix metabolism, proinflammatory factor phenotype, apoptosis, autophagy, and aging. On the other hand, the antioxidant proteins in the degenerated intervertebral disc tissue were significantly decreased, which significantly reduced the antioxidant capacity of the intervertebral disc tissue. These changes lead to a redox imbalance in disc cells, which are vulnerable to oxidative damage [64].

The pathophysiological role of oxidative stress on intervertebral disc degeneration is complex. More and more studies are devoted to elucidate the relationship between oxidative stress and intervertebral disc degeneration, and it is found that oxidative stress may be a key factor of intervertebral disc degeneration. Antioxidative stress therapy recognized as a promising treatment for disc degeneration [65]. However, in vitro experiments are insufficient to support the true effectiveness of these antioxidants in preventing or delaying human disc degeneration. Therefore, further clinical research is needed.

5. Limitations

Bibliometric analysis is widely used to measure the impact of articles in recent years. However, there are still some limitations. First, we only used the core collection of Web of Science (WOS) for searching literature. The more databases we use, the more information we can get and analyze. Other databases such as InCites and MEDLINE should be considered in future. Second, the main language of WOS is English. Articles written by other languages are excluded, which means some relevant articles to be not included. Third, citation number of each literature is time-dependent. Different time to search the articles, different citations may obtain. However, the trend of citation number of each literature is nearly the same.

This is the first research focusing on stem and progenitor cells in intervertebral disc by an analysis of the scientific landscape using bibliometric method. Our results can benefit scholars involved in the field of intervertebral disc degeneration by better understanding future research directions and trends. They can more specifically improve the state of treatment of intervertebral disc degeneration by paying more attention to adipose-derived mesenchymal stem cell and oxidative stress.

6. Conclusion

We demonstrated that research on stem and progenitor cells in intervertebral discs was in a rapid development stage. China had the most publications, and USA played a significant role with highest citations and H-index. The most high-yield author, organization, country, research directions, funds, and journals were Chen QX from Zhejiang University, Zhejiang University, China, Cell Biology, National Natural Science Foundation of China, and *Spine*, respectively.

Top 4 research hotspots contained "human intervertebral disc", "adipose-derived mesenchymal stem cell", "intervertebral disc degeneration", and "degenerative disc model". Meanwhile, research frontiers and trends were "microenvironment", "activation", "intervertebral disc degeneration", and "oxidative stress".

Our results can benefit researches by quickly grasp research hotspots and trends, which can provide a new perspective for further research.

Data Availability

The authors confirm that all data underlying the findings are fully available upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Yunzhong Cheng and Honghao Yang searched literatures, collected data, and prepared the manuscript. Yong Hai and Yuzeng Liu supervised and revised the manuscript. All authors have read and approved the content of the manuscript. Yunzhong Cheng and Honghao Yang are co-first authors of this article, contributing equally to the design and drafting of the manuscript.

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Research Article

Assessment of the Efficacy of Bone Marrow-Derived Mesenchymal Stem Cells against a Monoiodoacetate-Induced Osteoarthritis Model in Wistar Rats

Hadeer Mohamed Hamdalla[1], **Rasha Rashad Ahmed**[1], **Sanaa Rida Galaly**[1], **Osama Mohamed Ahmed**[2], **Ibrahim A. Naguib**[3], **Badrah S. Alghamdi**[4,5] and **Manal Abdul-Hamid**[1]

[1]*Cell Biology, Histology and Genetics Division, Department of Zoology, Faculty of Science, Beni-Suef University, P.O. Box 62521, Beni Suef, Egypt*
[2]*Physiology Division, Zoology Department, Faculty of Science, Beni-Suef University, P.O. Box 62521, Beni Suef, Egypt*
[3]*Department of Pharmaceutical Chemistry, College of Pharmacy, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia*
[4]*Department of Physiology, Neuroscience Unit, Faculty of Medicine, King Abdulaziz University, Jeddah 22252, Saudi Arabia*
[5]*Pre-Clinical Research Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia*

Correspondence should be addressed to Manal Abdul-Hamid; medo_bio@yahoo.com

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Osteoarthritis (OA) of the knee is a debilitating condition that can severely limit an individual's mobility and quality of life. This study was designed to evaluate the efficacy of bone marrow-derived mesenchymal stem cell (BM-MSC) treatment in cartilage repair using a rat model of monoiodoacetate- (MIA-) induced knee OA. OA was induced in the knee joint of rats by an intracapsular injection of MIA (2 mg/50 μL) on day zero. The rats were divided into three groups ($n = 6$): a normal control group, an osteoarthritic control group, and an osteoarthritic group receiving a single intra-articular injection of BM-MSCs (5×10^6 cells/rat). The knee diameter was recorded once per week. By the end of the performed experiment, X-ray imaging and enzyme-linked immunosorbent assay analysis of serum inflammatory cytokines interleukin-1beta (IL-β), IL-6, and tumor necrosis factor-α (TNF-α) and anti-inflammatory cytokines interleukin-10 and transforming growth factor-beta (TGF-β) were carried out. In addition, RT-PCR was used to measure nuclear factor-kappa B (NF-κB), inducible nitric oxide synthase (iNOS), and type II collagen mRNA levels and Western blot analysis was used to determine caspase-3 protein levels in all treated groups. Finally, hematoxylin/and eosin stains were used for histopathological investigation. Administration of BM-MSCs significantly downregulated knee joint swelling and MIA-induced (IL-1β, IL-6, and TNF-α) and upregulated IL-10 and TGF-β as well. Moreover, BM-MSC-treated osteoarthritic rats exhibited decreased expression of NF-κB, iNOS, and apoptotic mediator (caspase-3) and increased expression of type II collagen when compared to rats treated with MIA alone. The hematoxylin/eosin-stained sections revealed that BM-MSC administration ameliorated the knee joint alterations in MIA-injected rats. BM-MSCs could be an effective treatment for inflamed knee joints in the MIA-treated rat model of osteoarthritis, and the effect may be mediated via its anti-inflammatory and antioxidant potential.

1. Introduction

Knee osteoarthritis (OA) is a disorder that influences the musculoskeletal system in youth and the elderly [1]. It is a degenerative joint disease, which is marked by pain, erosion of articular cartilage, osteophytes, subchondral sclerosis, and a variety of biochemical and morphologic changes that occur in the synovial membrane and joint capsule [2]. The rapid increase of OA is expected to significantly impact health care and public health systems in the future. With an aging

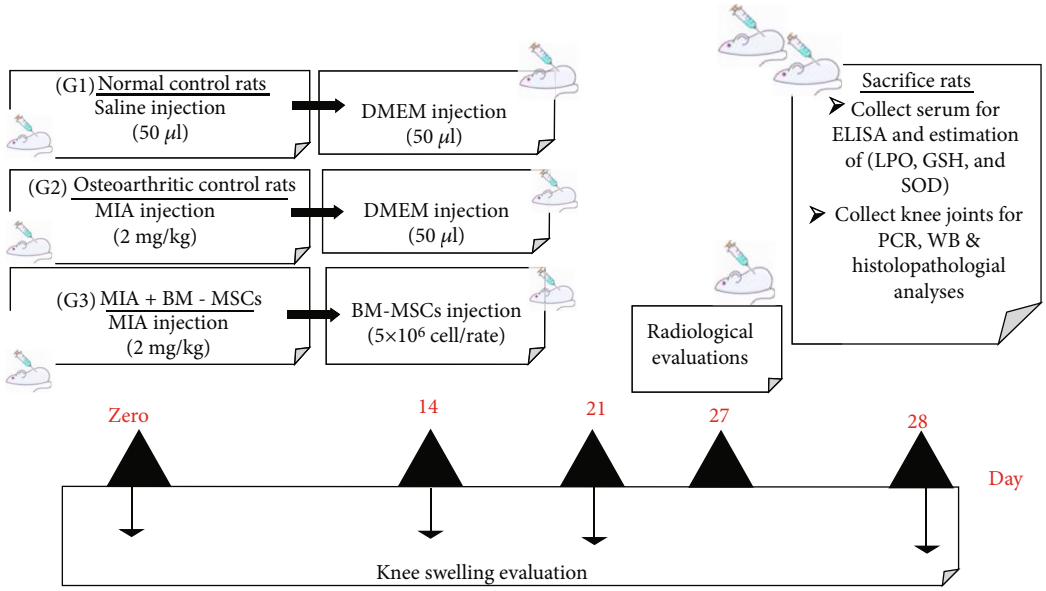

Figure 1: Timeline of the experiment depicting OA induction on zero day, treatment on the 14th day, and sacrifice of animals on the 28th day.

population, the physical and economic burden of OA is tremendous as it is considered one of the main reasons for disability among the elderly [1, 3].

The pathogenesis of diseases of joints such as OA is associated with progressive degeneration of articular cartilage, bone remodeling, and locally produced cytokines, chemokines, and other inflammatory mediators by synovium and chondrocytes. Up to date, it is not clear to determine strictly the exact cause of OA specifically and OA progression risk depends on a wide range of factors. The dysbacteriosis of the gut microbiota can be claimed to be a predisposing factor for OA pathogenesis since it leads to obesity, insulin resistance, and systemic inflammation [4]. However, most attempts at treatment have only been effective at reducing the disease symptoms (pain relief) [5].

Despite extensive work, there is currently no treatment that can cure or effectively slow the progression of OA [6]. Because the efficacy of new therapeutics is initially tested in animal models of OA [7], it is important to develop animal models that accurately depict joint pathogenesis and treatment response and provide useful biomechanical, radiological, and microscopic assessments of OA-affected tissues [8]. The monoiodoacetate- (MIA-) induced model of OA, as opposed to surgical models, is an ideal experimental model that is easy to generate, and it induces OA alterations similar to those observed in humans [9]. It exhibits increased inflammatory cytokines and decreased anti-inflammatory cytokines, thereby mimicking the inflammation process [10].

Current studies have focused on mesenchymal stem cell (MSC) therapies as they promote the protection, regeneration, and restoration of degenerated and injured joints resulting from arthritis [11–13]. Although the precise mechanism underlying the effectiveness of stem cell-based injectable treatments is not yet completely known [14], the capability of MSCs to migrate and engraft onto multiple musculoskeletal tissues and undergo differentiation into functional chondrocytes [15, 16], regenerate meniscus [17], and produce therapeutic growth factors and cytokines [6, 18] has drawn significant interest as a way to facilitate the repair of damaged tissues and halt disease progression. Consequently, the main objective of the presented work is to evaluate the ability of bone marrow-derived mesenchymal stem cells (BM-MSCs) to repair deterioration in the articular cartilage in MIA-induced osteoarthritis in a rat model utilizing radiographic, biochemical, and real-time polymerase chain reaction (RT-PCR), Western blot, and histological analyses.

2. Materials and Methods

The experiments were performed using eighteen adult male Wistar rats (weighing 130–150 g). They were brought from the animal house of Al-Nahda University, Beni Suef, Egypt, maintained under conditions of controlled humidity, fed with commercial rat pellets and water ad libitum, and their weight was measured weekly. All procedures followed the guidelines of the "experimental animal ethics committee" of the faculty of science, Beni-Suef University, Egypt, for the use and care of animals, and the ethical approval number is BSU/FS/2018/15.

2.1. Induction of Osteoarthritis (OA). The osteoarthritis model was constructed on day zero (Figure 1). The left knees of twelve rats were sprayed with 70% alcohol and then intra-articularly injected with 50 μL of sterile saline (0.9%) containing monosodium iodoacetate (MIA) (2 mg/50 μL) using a 21-gauge needle as previously described by Maresca et al. [19].

2.2. Isolation and Culture of Bone Marrow Mesenchymal Stem Cells. The protocol for the isolation and culture of BM-MSCs was done according to the procedure of Ahmed et al. [11] and Chaudhary and Rath [20]. BM-MSCs were flushed out of the humerus, femurs, and tibiae of the rats

and centrifuged at 3000 RPM for 5 min at room temperature. They were cultured in culture flasks containing Dulbecco's modified Eagle's medium (DMEM) (Life Science Group Ltd., UK) supplemented with 10% fetal bovine serum (Lonza Verviers Sprl, Belgium), 0.36% sodium hydrogen carbonate, and 1% penicillin/streptomycin (Life Science Group Ltd., UK) and kept at 37°C in a 5% CO_2 incubator. On the third day, the culture medium was changed. After 7–10 days, the cells were collected using trypsin (Greiner Bio-One, Germany).

2.3. Viability Assessment. For cell counting and viability assessment, the collected cells were washed and resuspended in DMEM. Then, 10 μL of 0.4% trypan blue was added to 10 μL of the cells and the mixture was counted on a hemocytometer. The BM-MSCs dispersed in DMEM with viability higher than 95% were immediately injected into the knee joint of osteoarthritic rats at a dose of 5×10^6 cells/rat.

2.4. Animal Grouping and Experimental Design. Eighteen adult male Wistar rats were randomly selected and categorized into the following three groups ($n = 6$) (Figure 1):

2.4.1. G1 (Normal Control Group). The rats within the control group received an intracapsular injection of saline (50 μL) and DMEM (50 μL) into the left knee joint at 0 and 14 days.

2.4.2. G2 (MIA-Induced OA Group). The rats within this group were administered a single intra-articular injection of 50 μL saline containing 2 mg MIA [19] and 50 μL of DMEM into the left knee joint at 0 and 14 days.

2.4.3. G3 (MIA + BM-MSC Group). On the 14^{th}-day post-MIA injection, the rats were treated using a single intra-articular injection (50 μL) of BM-MSCs at a dose of 5×10^6 cells/joint [21].

2.4.4. Knee Measurement. The differences in the measurements of the anterior-posterior diameters of the affected and unaffected knee joints were measured using a manual caliper [22]. The measurements were recorded on day zero and every week post-MIA injection until the end of the experiment. Then, the mean variance in the volume of injected knee edema (swelling) relative to the noninjected knee was obtained.

2.4.5. Radiographic Assessment (X-Ray). On day 27 post-OA induction and day 14 post-BM-MSC treatment, the knee joints of haphazardly taken rats under anesthesia were X-rayed (anterior-posterior position) using an X-ray apparatus (Shimadzu Corporation, Japan) to observe impairment in joint space, bone morphology, and response to BM-MSC treatment.

2.4.6. Blood and Tissue Samples. After 28 days, animals were anesthetized with diethyl ether inhalation and blood samples were collected from the jugular vein. Obtained blood was left to coagulate at ambient temperature for 30 min, followed by centrifugation at 3000 rpm for 15 min. Obtained serum was quickly removed and kept at −20°C until being used for the analysis of various biochemical parameters. Three knee samples from each group were fixed in 10% buffered formalin for histopathological evaluation, whereas the others were kept at −20°C until being utilized for RT-PCR and Western blot analysis.

2.4.7. Enzyme-Linked Immunosorbent Assay Analysis. The amounts of serum tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), IL-6, IL10, and transforming growth factor-beta (TGF-β) of all groups were defined using specific enzyme-linked immunosorbent assay kits supplied by MyBioSource (USA) according to the manufacturer's instructions.

2.4.8. Antioxidant Defense System and Oxidative Stress Analysis. The glutathione (GSH) content, lipid peroxidation, and superoxide dismutase (SOD) activity were measured in serum as part of the antioxidant defense system. Later on, lipid peroxidation was determined following the method of Preuss et al. [23] based on the determination of malondialdehyde (MDA), which is an end product of lipid peroxidation reacting with thiobarbituric acid (TBA) to yield a pink-colored TBA-reactive substance, which assesses the amount of lipid peroxidation. SOD activity was determined following the Nishikimi et al. [24] procedure. This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitro blue tetrazolium dye. GSH levels were measured following the method of Beutler et al. [25]. It is the reduction of 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) by the thiol group (SH) which is present in GSH to form 5-thio-2-nitrobenzoic acid, where the latter can be assayed colorimetrically.

2.4.9. Real Time-PCR (RT-PCR) Analysis. The QIAGEN tissue extraction kit (QIAGEN, USA) was used for total RNA isolation. Then, 0.5–2 μg of total RNA was used for cDNA synthesis using a kit from Fermentas (USA). An Applied Biosystem instrument with software version 3.1 (StepOne™, USA) was for real-time qPCR amplification and analysis. The qPCR assay was done with primer sets optimized for the annealing temperature. The sequences of the primers are presented in Table 1.

2.4.10. Western Blot Analysis. Western blot (WB) analysis was implemented to assess the amount of protein of NF-κB p50, NF-κB p65, caspase-3, and cleaved caspase-3 in the knee samples. Briefly, the proteins were extracted from the left knee joints ($n = 3$) using ice-cold radioimmunoprecipitation assay buffer (RIPA buffer) supplemented with protease and phosphate inhibitors (Bio Basic Inc., Canada). Equivalent amounts of protein (30 μg) were isolated on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Furthermore, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% skim milk in TBS containing Tween 20 overnight. The membranes were incubated at 4°C with primary antibodies against NF-κB p50, NF-κB p65, caspase-3, and cleaved caspase-3. Following washing with TBST, membranes were incubated with the corresponding secondary antibodies and developed using an enhanced chemiluminescence kit (BioRad, USA). Finally, the developed blots were scanned and band intensity was measured using ImageJ software (NIH, USA).

Table 1: Primer sequences used for real-time PCR.

Target gene	Primer sequence
NF-κB	Forward primer: 5′-CATTGAGGTGTATTTCACGG-3′ Reverse primer: 5′-GGCAAGTGGCCATTGTGTTC-3′
iNOS	Forward primer: 5′-GACCAGAAACTGTCTCACCTG-3′ Reverse primer: 5′-CGAACATCGAACGTCTCACA-3′
Type II collagen	Forward primer: 5′-GAGTGGAAGAGCGGAGACTACTG-3′ Reverse primer: 5′-CTCCATGTTGCAGAAGACTTTCA-3′
Beta-actin	Forward primer: 5′-TGTTTGAGACCTTCAACACC-3′ Reverse primer: 5′-CGCTCATTGCCGATAGTGAT-3′

2.4.11. Histopathology of the Knee Joint. After dissection, 3 knee joint samples from each group were rapidly excised and trimmed for histopathological examination. They were fixed in 10% buffered formalin for 24 h and decalcified in 10% formic acid solution, dehydrated, and embedded in paraffin wax. After cutting into 5 μm sections, the slides were stained with hematoxylin and eosin stain for examination by light microscopy. The histopathology of OA is graded using the modified Mankin grading system [26, 27] as follows: the cartilage structure was scored from 0 to 4 (Table 2), where 0 is normal, 1 is surface irregularities, 2 is complete disorganization, 3 is clefts into the noncalcified cartilage layer, and 4 is clefts into the calcified cartilage layer. Furthermore, cellular abnormalities were scored on a scale of 0 to 3, where 0 is normal, 1 is hypercellularity, including small superficial clusters, 2 is clusters, and 3 is hypocellularity. Finally, tidemark was graded on a scale of 0 to 1, where 0 is intact and 1 is damaged.

2.4.12. Statistical Analysis. All the data were presented as the mean ± SEM, where $P < 0.05$ was deemed statistically significant. All values were analyzed by IBM-SPSS software (version 25.0) using one-way ANOVA followed by post hoc Dunnett's t-test at different variance levels.

3. Results

3.1. Effect of BM-MSCs on Knee Diameter Measurements. The MIA-treated group showed an increase in knee diameter during the first two weeks post-MIA injection compared with the values on day zero before MIA injection. However, MIA rats treated with BM-MSCs on day 14 exhibited a reduction in the knee measurement throughout the third and fourth weeks of the experiment compared to the MIA-treated group (Figure 2).

3.2. Effect of BM-MSCs on Radiographic Changes. At the end of the treatment period, the anterior and posterior views of the left knee joints from all groups were X-rayed. Compared with those of the normal rats (Figure 3(a)), the knees of the MIA-treated group showed OA alterations, such as cartilage degradation as evidenced by the existence of bone erosions, a narrow joint space, and minute marginal osteophytes (Figure 3(b)). In contrast, the BM-MSC-treated knees exhibited a significant restoration of normal joint morphology, marked by the recovery of joint space narrowing and disappearance of osteophytosis osteophytotic (Figure 3(c)).

Table 2: The modified Mankin score of microscopic observation of OA articular cartilage.

Category	Subcategory	Score
Structure	Normal	**0**
	Surface irregularities	**1**
	Complete disorganization	**2**
	Clefts to the noncalcified layer	**3**
	Clefts to calcified layer	**4**
Cells	Normal	**0**
	Hypercellularity	**1**
	Hypocellularity	**2**
	Pyknosis	**3**
Tidemarks	Intact	**0**
	Damaged	**1**

3.3. ELIZA Evaluation

(a) Effect of BM-MSCs on the serum proinflammatory cytokines TNF-α, IL-1β, and IL-6

A remarkable increase ($P < 0.05$) in the serum levels of TNF-α, interleukin-1 beta (IL-1β), and IL-6 (Table 3) was observed in the MIA-treated group in comparison with the normal control group. On the contrary, the MIA + BM-MSC group had a significant decrease ($P < 0.05$) in the serum levels of TNF-α, IL-1β, and IL-6 in comparison with the MIA group.

(b) Effect of BM-MSCs on serum IL-10

The serum levels of IL-10 (Table 3) were markedly reduced in the osteoarthritic control group in comparison with the normal control group ($P < 0.05$), whereas the serum levels of IL-10 were remarkably increased ($P < 0.05$) in the BM-MSC-treated group compared with the MIA group.

(c) Effect of BM-MSCs on serum transforming growth factor-beta (TGF-β)

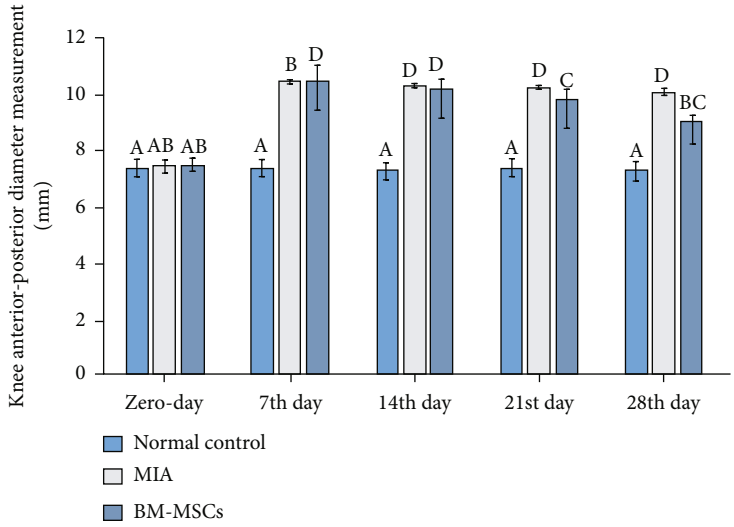

Figure 2: Knee anterior-posterior diameter measurements in normal control, MIA, and MIA + BMMSC groups. At each period, the means, which have different symbols (letters), are significantly different at $P < 0.05$.

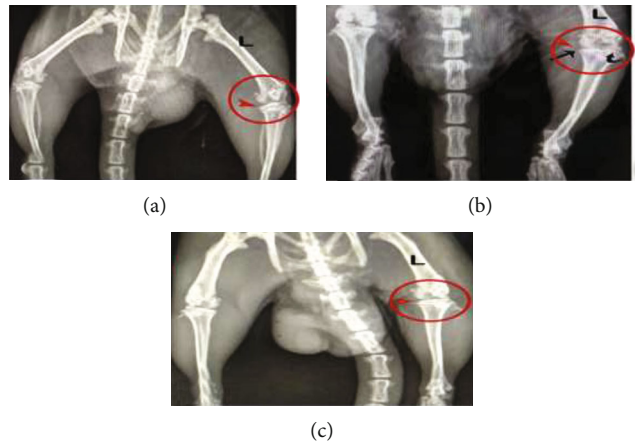

Figure 3: Radiographic changes in the left knee joint (L). (a) The normal control group shows a healthy knee joint with a smooth articular cartilage surface and normal joint space (arrowhead). (b) The MIA-treated group demonstrates OA alternations such as a remarkable narrowing of the joint space (arrowhead), erosion, subchondral sclerosis (curved arrow), and osteophyte (black arrow). (c) The MIA + BMMSC-treated group shows nearly restored normal joint space (arrowhead) and cartilage surface.

Table 3: Effect of BM-MSCs on serum levels of TNF-α, IL-6, IL-10, and TGF-β of MIA-induced OA.

Groups	Parameters				
	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)	TGF-β (pg/mL)
Normal control	22.16 ± 1.62^{a}	8.58 ± 3.14^{a}	60.06 ± 2.97^{a}	320.19 ± 2.33^{c}	114.49 ± 0.52^{a}
MIA	95.033 ± 0.71^{c}	130.39 ± 3.95^{c}	178.40 ± 5.86^{c}	119.08 ± 3.71^{a}	260.65 ± 6.62^{c}
MIA + BM-MSCs	60.40 ± 2.05^{b}	2.21 ± 4.95^{b}	130.13 ± 8.50^{b}	288.36 ± 1.54^{b}	134.98 ± 3.88^{b}

The number of samples in each group is six and the data are described as means ± SEM. For each parameter, means, which have different superscript symbols, are statistically significant, $P < 0.05$.

MIA administration had significantly increased the serum levels of TGF-β (Table 3) compared with the normal control rats. In contrast, the MIA + BM-MSC-treated group showed a reduction ($P < 0.05$) in the levels of TGF-β compared with the MIA-treated group and an increase relative to the normal rats.

3.4. Effect of BM-MSCs on Serum Levels of Malondialdehyde (MDA), Superoxide Dismutase (SOD), and Glutathione (GSH). MIA-induced osteoarthritic rats had significantly ($P < 0.05$) increased MDA levels and decreased the activity of SOD and the concentration of GSH (Table 4). On the other hand, BM-MSC-treated rats exhibited a significant

Table 4: Effect of BM-MSCs on serum levels of MDA, GSH, and the activity of SOD of MIA-induced OA.

Groups	Parameters		
	MDA (nmol/mL)	GSH (mg/dL)	SOD (U/mL)
Normal control	0.016 ± 0.004^{a}	183.96 ± 16.26^{c}	339.46 ± 13.49^{c}
MIA	0.162 ± 0.037^{c}	16.057 ± 2.9^{a}	150.97 ± 11.08^{a}
MIA + BM-MSCs	0.087 ± 0.006^{b}	55.33 ± 5.98^{b}	227.68 ± 8.33^{b}

The number of samples in each group is six and the data are described as means ± SEM. For each parameter, means, which have different superscript symbols, are statistically significant, $P < 0.05$.

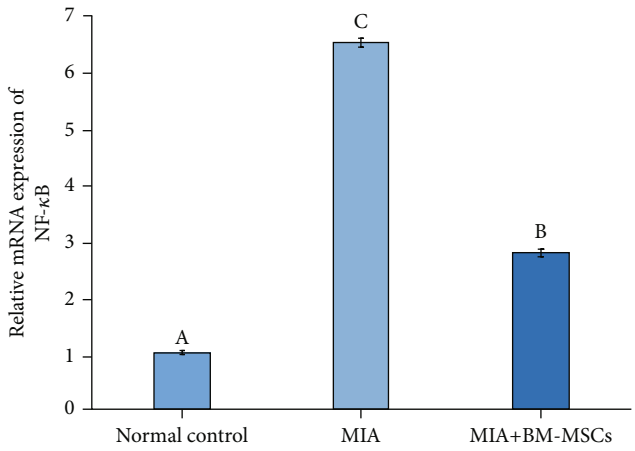

Figure 4: Effect of BM-MSC treatment (5×10^6 cells/rat) on the mRNA expression level of NF-κB in MIA-induced animals. Means, which have different symbols, are significantly different at $P < 0.05$.

decrease in MDA levels accompanied by an elevation in the activity of SOD and the content of GSH.

3.5. Effect of BM-MSCs on mRNA Expression Levels of Nuclear Factor-Kappa B (NF-κB), Inducible Nitric Oxide Synthase (iNOS), and Type II Collagen. The role of BM-MSC administration in osteoarthritic rats on the expression of nuclear factor-kappa B (NF-κB), inducible nitric oxide synthase (iNOS), and type II collagen was determined by qRT-PCR (Figures 4–6), respectively. MIA administration markedly increased the expression of NF-κB and iNOS in comparison with the normal control group. On the contrary, the BM-MSC treatment of osteoarthritic rats significantly inhibited the expression levels of NF-κB and iNOS compared with the group treated with MIA only. On the other hand, BM-MSCs enhanced the expression of collagen type II mRNA compared to that of the osteoarthritic control group, which exhibited low expression of collagen type II.

3.6. Effect of BM-MSCs on the Protein Expression Levels of NF-κB p50 and NF-κB p65. Western blot analysis demonstrated the MIA-induced increase in the protein expression levels of NF-κB p50 and NF-κB p65 (Figure 7) in the knee joints of osteoarthritic rats relative to the normal knee joints, whilst MIA + BM-MSC-treated knee joints showed significant reduction in protein expression levels of NF-κB p50 and NF-κB p65 compared with the MIA-treated knee joints without any treatment.

3.7. Effect of BM-MSCs on the Protein Expression Levels of Caspase-3 and Cleaved Caspase-3. Western blot analysis revealed that the protein levels of cleaved caspase-3 and caspase-3 (Figure 8) were markedly enhanced in the knee joints of osteoarthritic rats in comparison with the normal rats. Furthermore, the upregulated protein levels of caspase-3 and cleaved caspase-3 were significantly attenuated in knee joints of osteoarthritic rats treated with BM-MSCs compared with the osteoarthritic control group.

3.8. Effect of BM-MSCs on Histopathological Changes. Hematoxylin and eosin sagittal stained sections of the normal knee revealed a normal histological composition of the joint capsule, articular cartilage, and subchondral bone as well as intact tidemarks (Figure 9(a)). In contrast, the stained sections of the osteoarthritic rats (MIA group) showed significant histopathological alterations in the cartilage including a reduction in thickness, clefting, uneven articular surface, and degenerated chondrocytes accompanied by apoptosis (Figure 9(b)). Osteoarthritic rats (Figure 9(b)) also displayed bone destruction in the discontinuous thin cancellous bone trabeculae with blind ends and widening of the bone marrow space, which contained fewer hematopoietic cells as well as invisible tidemarks. Moreover, matrix changes included a severe loss, degeneration, and heterogeneous distribution of chondrocytes in the growth plate (Figure 9(c)). However, osteoarthritic rats treated with BM-MSCs exhibited no histopathological bone lesions. In contrast, osteoarthritic rats treated with BM-MSCs showed profound protection against OA-related articular cartilage defects and indicated no histopathological lesions in cartilage or bone compared with osteoarthritic knee joints (Figures 9(d) and 9(e)). Likewise, the total score of the modified Mankin system (Table 5) was significantly lower in the BM-MSC osteoarthritic-treated group ($P < 0.05$) in comparison with the osteoarthritic control group which confirms the protective properties of stem cell treatment against further destruction of the cartilage in OA knee joints.

4. Discussion

OA was considered for a long time as a noninflammatory wear and tear condition involving cartilage degeneration. However, it has become clear that it is a whole-joint disease in which catabolic processes cause cartilage degradation and inflammation, which plays a key role in the pathogenesis and development of OA [28].

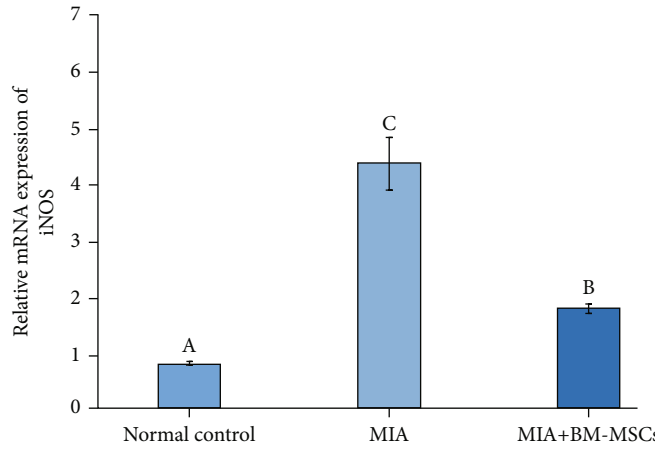

Figure 5: Effect of BM-MSC treatment (5×10^6 cells/rat) on the mRNA expression level of iNOS in MIA-induced osteoarthritic animals. Means, which have different symbols, are significantly different at $P < 0.05$.

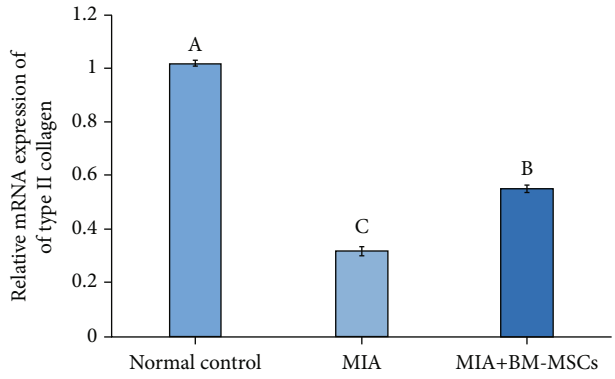

Figure 6: Effect of BM-MSC treatment (5×10^6 cells/rat) on the mRNA expression level of type II collagen in MIA-induced osteoarthritic animals. Means, which have different symbols, are significantly different at $P < 0.05$.

Considering the limited reparability of cartilage [16, 29] and that no cure is currently available for OA [30], MSCs are regarded as an auspicious candidate for knee OA treatment because of their chondroprotective effects, chondrogenic potential, and paracrine effects [31–33], as well as their ability to enhance the production of various extracellular matrix (ECM) components [34, 35].

In the present study, we shed light on the possible underlying mechanisms of action of a single intra-articular injection of BM-MSCs as therapy for cartilage damage in an MIA-induced OA rat model (Figure 10).

Joint swelling is frequent in various kinds of arthritis and is driven by edema, which occurs as a result of leakage of fluid from endothelial cells of the blood vessels into the inflamed synovium [36]. The intrainjection of BM-MSCs resulted in a substantial reduction in the increased values of the left knee diameter post-MIA administration. Similarly, a study by Kehoe et al. [37] revealed that treatment with BM-MSCs reduced knee swelling, which was ascribed to alterations in permeability of synovial endothelial cells to soluble substances produced by the MSCs.

Our radiographic findings in harmony with de Morais et al. [8] and Jaleel et al. [38] illustrated that MIA-induced chondral injury along with inflammation resulted in osteophytosis, bone sclerosis, and a reduction in joint space, analogous to human osteoarthritis. Moreover, radiography images revealed that two weeks of treatment with BM-MSCs attenuated the effect of MIA and resulted in a direct regenerative effect on knee joint cartilage.

Because damaged cartilage is subjected to a progressive inflammatory environment [39], several inflammatory signaling pathways, including the nuclear factor-kappa B (NF-κB), have been implicated in the control of OA [40].

The classical-canonical pathway of NF-κB is stimulated in chondrocytes and synoviocytes of articular joints by mechanical stress or cytokines (IL-1β and TNF-α) (Figure 10). It is started with the activation of IB kinase (IKK), resulting in phosphorylation and degradation of IκBα by the proteasome, and then, NF-κB p65 as well as NF-κB p50 protein is released and translocated from the cytoplasm to the nucleus [41].

Activated chondrocytes and synoviocytes subsequently produce a plethora of inflammation-related factors, including matrix metalloproteinase proteins, inducible nitric oxide synthase (iNOS), IL-1β, IL-6, and TNF-α, and these cytokines further activate the signaling cascade [42].

Our results indicated that BM-MSCs significantly suppressed NF-κB p50, NF-κB p65, TNF-α, IL-1β, and IL-6 in osteoarthritic rats (Figure 10). These findings are consistent with Mancuso et al. [43] and Wang et al. [44]. A study by Wang et al. [45] hypothesized that MSC administration displayed anti-inflammatory effects via lessening excess TNF-α (an activator of NF-κB) and blocking the phosphorylation of the NF-κB p65 subunit in spinal cord injury.

Moreover, BM-MSC treatment significantly elevated the serum levels of IL-10 which is considered an anti-inflammatory cytokine that possesses chondroprotective characteristics [46]. Moreover, it can induce the proliferation of chondrocytes [47] and ameliorate the severity of arthritis and cartilage degeneration [48]. The immunomodulatory capacity of activated MSCs alters inflammatory

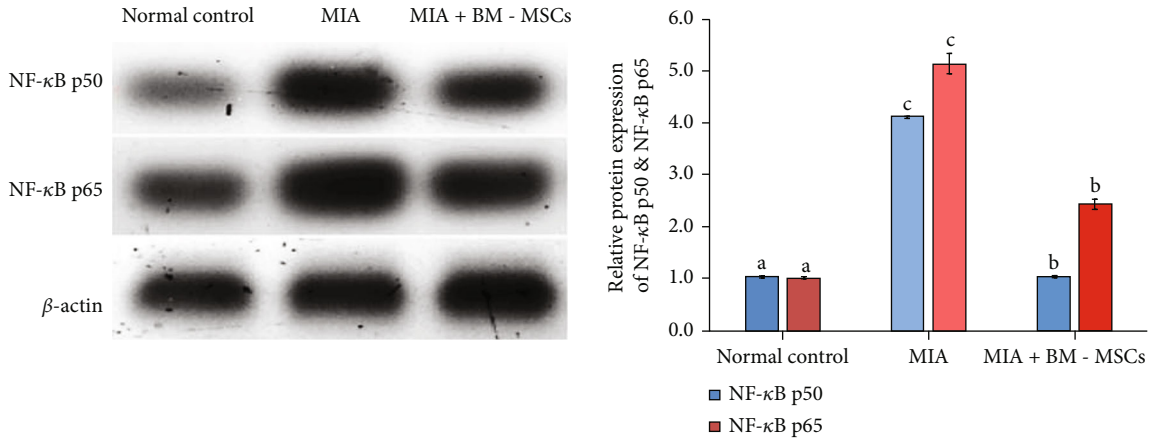

Figure 7: Effect of BM-MSC treatment (5×10^6 cells/rat) on the protein levels of NF-κB p50 and NF-κB p65 in MIA-induced animals. Means, which have different symbols, are significantly different at $P < 0.05$.

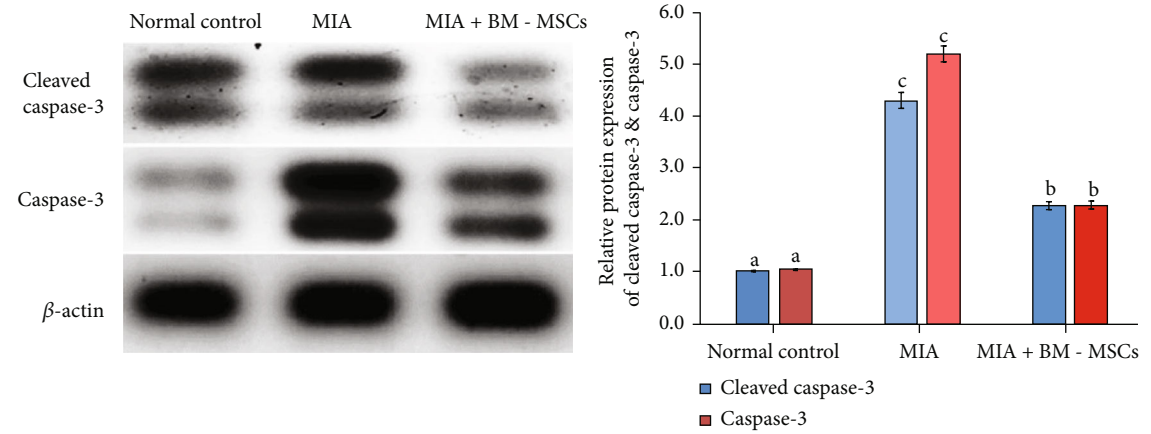

Figure 8: Effect of BM-MSC treatment (5×10^6 cells/rat) on the protein levels of cleaved caspase-3 and caspase-3 in MIA-induced animals. Means, which have different symbols, are significantly different at $P < 0.05$.

cytokine levels during OA and can affect IL-10 expression, which results in tissue survival [49]. Previous studies proposed that prostaglandin E2 (PGE2) released by MSCs can increase the secretion of IL-10 by engaging the E-type prostanoid receptors, EP2 and EP4 receptors, on M2 macrophages which eventually repair the damage of the cartilage [44, 50].

In our study, BM-MSC injection also markedly decreased inducible nitric oxide synthase expression in the osteoarthritic knee joints. Hamilton et al. [51] stated that intra-articular injection of MSC could downregulate the iNOS level in macrophages and eventually reduces the generation of M1 macrophages.

The expression of proinflammatory and damaging mediators of OA, such as the iNOS gene, has also been linked to NF-κB signaling [52, 53]. iNOS is considered an enzyme responsible for the generation of nitric oxide (NO). Excessive production of NO by iNOS appears to be involved in OA pathogenesis through modulating ECM homeostasis and cytokine expression, which results in oxidative damage and chondrocyte apoptosis [54].

The immunosuppressive nature of MSCs may also explain the outcomes of iNOS levels in our study. Under inflammatory conditions, interferon γ (IFNγ), in combination with one of three additional proinflammatory cytokines, TNF-α, IL-1, or IL-1β, induces the immune activity of MSCs. In response to this cytokine combination, MSCs express multiple chemokines and iNOS, which directly prevent the proliferation and function of T cells [55].

Even though transforming growth factor-beta (TGF-β) signaling has a principal role in cartilage development and in maintaining articular chondrocyte homeostasis in synovial joints, in the present study, TGF-β is potentially involved in joint degeneration. Similarly, a study by Dranitsina et al. [56] revealed that MIA-OA causes an increase in the expression of Tgfb1 genes in rat cartilage cells. Our results, in contrast to Halfaya et al. [57], indicated a significant rise in the level of transforming growth factor β (TGF-β) in OA joints compared with that of the control group [46]. Van der Kraan [28] postulated that an elevation of the TGF-β level could activate inflammation that may be involved in OA pathogenesis by altering

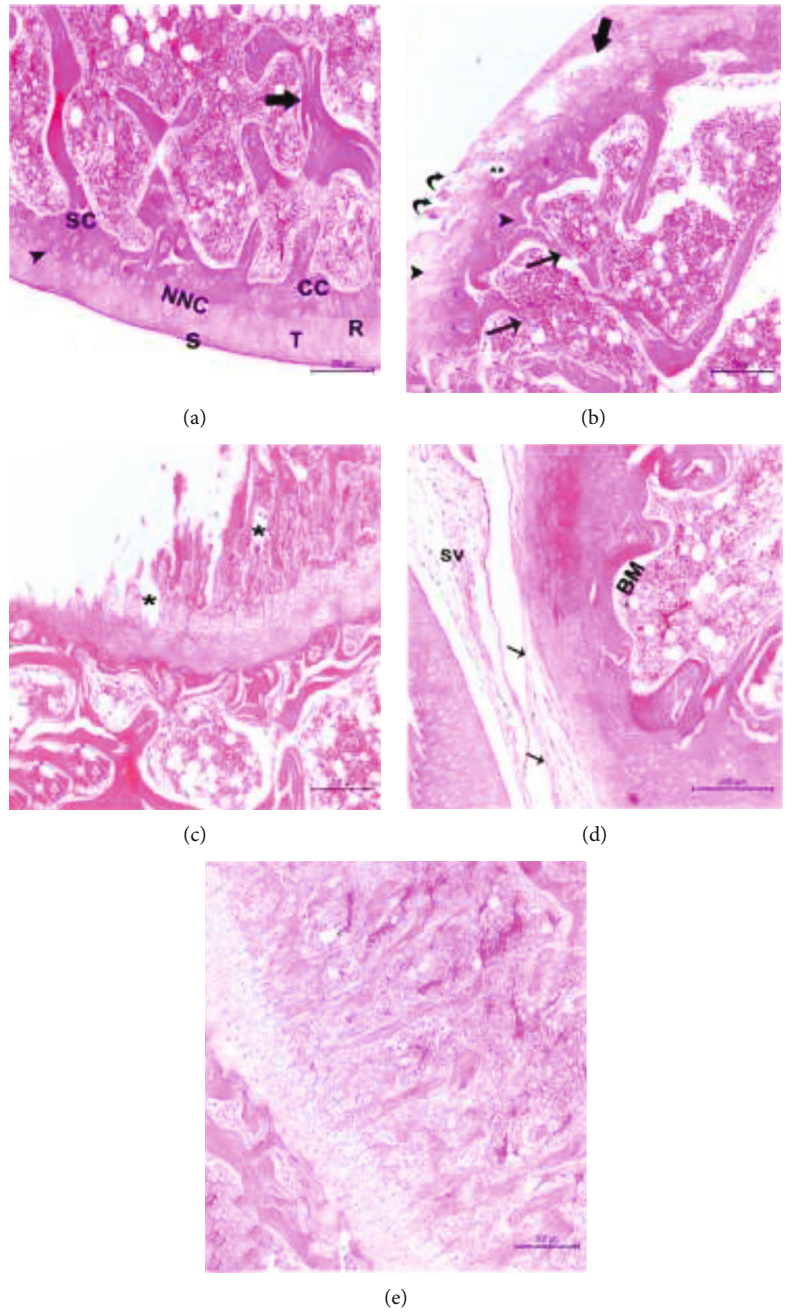

Figure 9: Photomicrographs of hematoxylin and eosin- (H&E-) stained sections of left knees joints of the following groups: (a) is a section from the control group which demonstrates the normal structure of articular cartilage, with a regular smooth intact surface and well-organized chondrocytes, which appeared in noncalcified (NCC) and calcified (CC) regions of cartilage with a clear intact tidemark (arrowhead) in between. The noncalcified region (NCC) of the articular cartilage is arranged in three zones: superficial (S), transitional (T), and radial (R) zones. It also shows intact subchondral bone (SC) with well-oriented bony trabeculae (arrow) (scale bar = 200 μm). (b, c) Are sections from osteoarthritic rats (MIA-treated group). (b) Depicts clefting (curved arrows), surface erosion, degeneration of the surface layer with discontinuity of the matrix (star), discontinuous thin cancellous bone trabeculae having blind ends (arrows), decrease in thickness of articular cartilage, degenerated chondrocytes with pyknotic nuclei (arrowheads), and widening of bone marrow space (BM) containing less hemopoietic cells, invisible tidemark, and large, thickened area at the joint margin and disorganization of the articular cartilage with some cell clusters (thick arrow). (c) Shows the matrix change, loss, degeneration (∗), and heterogeneous distribution of chondrocytes in the growth plate. (d, e) Are sections from the treatment group (MIA + BMMSCs). (d) Displays intact synovial membrane (SV) and marked restoration of the normal structure of the articular cartilage intact surface and increase in its thickness and nearly normal bone marrow space (BM) compared to the MIA-treated group, organized fibrous connective tissue (thin arrows), clarification of tidemark (arrowhead), and cell layers with few shrunken chondrocytes, some empty lacunae (scale bar = 200 μm). (e) Demonstrates neatly and normally oriented chondrocytes of the growth plate (scale bar = 200 μm).

Table 5: Mankin scoring of the cartilage among experimental groups.

Groups	Parameters			
	Cartilage structure	Cellularity	Tidemarks	Overall Mankin score
Normal control	0 ± 0	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
MIA	4.0 ± 1.41^{c}	1.26 ± 0.52^{c}	1.0 ± 0.0^{c}	11.17 ± 1.28^{c}
MIA + BM-MSCs	2.50 ± 0.76^{b}	0.55 ± 0.22^{b}	0.33 ± 0.21^{c}	4.17 ± 0.60^{b}

The number of samples in each group is six and the data are described as means ± SEM. For each parameter, means, which have different superscript symbols, are statistically significant, $P < 0.05$.

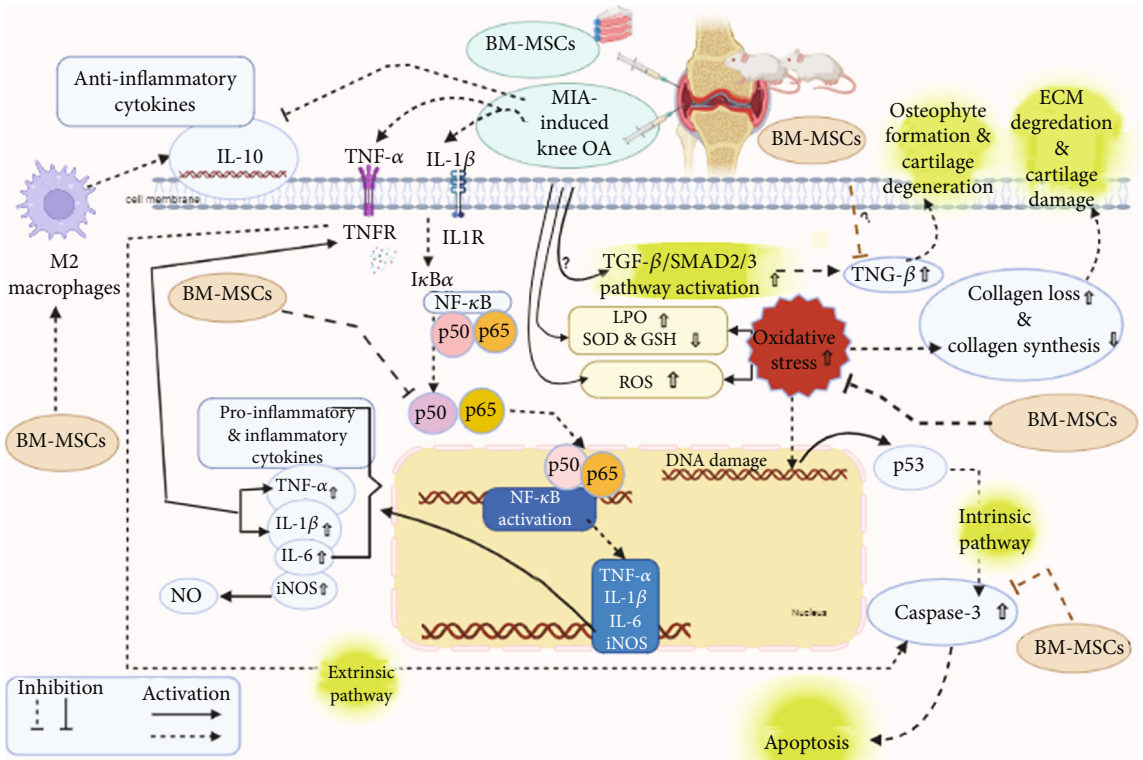

Figure 10: The therapeutic effect of BMMSCs on cartilage damage due to inflammation, extracellular matrix (ECM) degradation, and apoptosis in MIA-induced knee osteoarthritis.

cellular differentiation and causing joint deterioration. Additionally, studies have reported that TGF-β signaling mediated by Smad2/3 may be involved in OA progression by inducing the recruitment of MSCs and osteoprogenitors to the subchondral bone, ending with aberrant bone remodeling that initiates and worsens osteoarthritis. Nevertheless, the activation and catabolic role of TGF-β in OA requires further investigation.

However, TGF-β levels after BM-MSC treatment were still higher compared with those of the normal control group. Studies have reported that MSCs could inhibit T-cell proliferation and promote apoptosis in T cells, ending with fragments that trigger phagocytes to release TGF-β [6, 58].

Apoptosis also plays a key role in OA pathophysiology. MIA has been shown to have necrotic and proapoptotic effects on rat chondrocytes in vitro, whilst TNF-α activates the tumor necrosis factor receptor (TNFR) or death receptors which eventually triggers the extrinsic pathway of apoptosis (Figure 10).

Korotkyi et al. [59] demonstrated that MIA-OA induced free radical reactions that caused the accumulation of superoxide anion radicals, hydrogen peroxide, and NO and thiobarbituric acid-reactive compounds that are intermediate products of lipid peroxidation. Elevated oxidative stress and ROS levels triggered by MIA led to the activation of the intrinsic pathway through the depolarization of membrane potential, the promotion of the discharge of cytochrome c, and the activation of caspase-3 [10, 60, 61]. Caspase-3 contributes to the overall apoptotic process by cleaving various cellular substrates [62]. Korotkyi et al. [59] also mentioned that MIA-induced OA led to a decrease in superoxide dismutase (SOD) activity of glutathione (GSH) which represents the first line of antioxidants that catalytically scavenge the free radicals [13].

Overall, MIA-OA condition results in an imbalance between the intensity of the formation of free radicals and their neutralization by the antioxidant defense system. Therefore, inhibiting ROS and caspase-3 expression in OA could potentially inhibit apoptosis.

In this context, we tested the effect of BM-MSC injection on the increase of the lipid peroxidation product (MDA) and the decrease of SOD and GSH levels in MIA-induced OA in the knee joints of the rat. Our data showed that the antioxidant system was boosted and that the increased level of caspase-3 and MDA in OA knee joints was ameliorated following intra-articular MSC treatment.

MSCs release an array of paracrine molecules, known as secretome, consisting of a variety of proteins with diverse biological functions, including immune regulation, antiapoptotic effects, and antioxidative effects. Antioxidant effects exhibited by MSCs and their secretome are attributed to their ability to scavenge free radicals, upregulate the antioxidant defense system, and alter cellular bioenergetics [63]. MSC immunosuppressive capabilities can also prevent the production of ROS and lower oxidative stress. Most recently, BM-MSCs decreased oxidative stress and enhanced antioxidant activity in severe acute pancreatitis in rats by inducing the nuclear translocation of nuclear factor erythroid 2-related factor 2, an emerging regulator of cellular resistance to oxidants, via the PI3K/AKT signaling pathway [64]. Meanwhile, He et al. [65] proposed that the paracrine effect of MSC mitigated ischemia-induced apoptosis by increasing the Bcl-2-to-Bax ratio and inhibiting the activation of caspase-3.

Oxidative stress and ROS have also been linked to OA pathophysiology by inhibiting new cartilage extracellular material (ECM) synthesis, leading to a loss of integrity of the cartilage [66]. The degradation and low accumulation of type II collagen, a predominant component of ECM that interacts with proteoglycans, supplying the cartilage with the elasticity and capacity for deformation, have been implicated in OA condition [67]. Besides, the newly produced molecules to compensate for the loss are often damaged which inhibits cartilage repair [68].

Lepetsos and Papavassiliou [54] suggested that ROS restrains mitochondrial oxidative phosphorylation and ATP formation in cultured chondrocytes, which eventually decreases the synthesis of collagen and proteoglycans and results in cartilage degradation.

On the other hand, MSCs have also been shown to promote chondrogenesis by replenishing the ECM of articular cartilage [35]. Intra-articular BM-MSC administration diminished the loss of collagen type II in OA knee joints. Ahmed et al. [13] have suggested that BM-MSCs could promote the antioxidant defense system at the expense of the oxidative stress in tissues, hence, inhibiting the subsequent inflammatory process (Figure 10).

Histopathological evaluation was the major endpoint examined in the current study. Four weeks post-MIA administration, the osteoarthritic control displayed multiple histopathological changes in the knee joint including severe damage to the cartilage structure which was manifested by a loss of integrity, clefts, degeneration of the surface layer, matrix changes, dispersed and pyknotic chondrocytes, and hypocellularity resulting from the loss and degeneration of chondrocytes [69, 70]. In the present study, a single intra-articular injection of BM-MSCs significantly lessened the inflammation and provided adequate protection against MIA-induced histopathological alterations, which were demonstrated by the preserved structure of articular cartilage, ECM, and the underlying subchondral bone [21, 71], However, the impact of a single injection of BM-MSC on cartilage regeneration and proliferation should be further studied. These results are also supported by the overall Mankin score as the rats, which received an intra-articular injection of BM-MSC showing a remarkable amelioration of the articular cartilage structure represented by a lower Mankin score compared with the osteoarthritic rats without treatment.

Considering the biochemical, molecular, and histopathological outcomes, our results suggest that BM-MSC treatment regulates and reduces OA-induced inflammation, postpones cartilage degradation, and promotes cartilage regeneration through paracrine activity [72].

Although this study has reached its aims, there were some potential limitations that the relevant mechanism underlying the effects of BM-MSCs on OA has not yet been further confirmed because of an insufficient small sample size and a lack of ability in predicting the pathway and safety in clinical investigations. In future studies of OA and treatments with BM-MSCs, researchers should focus on in-depth investigations of the various molecular mechanisms underlying OA and screening and identifying specific signaling pathways.

5. Conclusion

It was concluded that intra-articular injections of BM-MSCs significantly enhanced the radiological, biochemical, molecular, and histopathological outcomes of rats suffering from knee OA induced by MIA over a two-week period. However, cartilage regeneration probably takes a long time to develop. Therefore, to determine the long-term efficacy of BM-MSCs on the progress of knee OA, long-term studies should be carried out.

Abbreviations

BM-MSCs: Bone marrow-derived mesenchymal stem cells
DMEM: Dulbecco's modified Eagle's medium
DTNB: 5,5′-Dithiobis-2-nitrobenzoic acid
ECM: Extracellular matrix
MDA: Malondialdehyde
GSH: Reduced glutathione
IFNγ: Interferon γ
IL: Interleukin
iNOS: Inducible nitric oxide synthase
MIA: Monosodium iodoacetate
NF-κB: Nuclear factor-kappa B
NO: Nitric oxide
OA: Osteoarthritis
PGE2: Prostaglandin E2
ROS: Reactive oxygen species
TBA: Thiobarbituric acid
TGF-β: Transforming growth factor-beta
TNFR: Tumor necrosis factor receptors
TNF-α: Tumor necrosis factor-α.

Data Availability

This published article includes all of the data generated or analyzed during this investigation.

Ethical Approval

All experimental procedures were performed in accordance with recommendations, instructions, and guidelines stated by the ethics committee for care and use of animals, faculty of science, Beni-Suef University, Egypt (ethical approval number: BSU/FS/2018/15).

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

RRA, SRG, and MA conceived and designed the experiments. HMH performed the experiments and analyzed the data. IAN and BSA and OMA provided experimental technical support and assisted in completing the study at different stages. HMH drafted the manuscript. RRA, SRG, and MA finalized the paper. All authors are in agreement with the contents of the manuscript. All authors read and approved the final manuscript.

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Review Article

The Regulatory Role of Ferroptosis in Bone Homeostasis

Yuan Xiong,[1,2] **Lang Chen**,[1,2] **Ze Lin**,[1,2] **Yiqiang Hu**,[1,2] **Adriana C. Panayi**,[3] **Wu Zhou**,[1,2] **Yun Sun**,[4] **Faqi Cao**,[1,2] **Guodong Liu**,[5] **Guangdong Dai**,[6] **Bobin Mi**,[1,2] **and Guohui Liu**[1,2]

[1]*Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*
[2]*Hubei Province Key Laboratory of Oral and Maxillofacial Development and Regeneration, Wuhan 430022, China*
[3]*Department of Plastic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02152, USA*
[4]*Department of Neurosurgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*
[5]*Medical Center of Trauma and War Injuries, Daping Hospital, Army Medical University, Chongqing 400042, China*
[6]*Pingshan District People's Hospital of Shenzhen, Pingshan General Hospital of Southern Medical University, Shenzhen, Guangdong, China 518118*

Correspondence should be addressed to Bobin Mi; mibobin@hust.edu.cn and Guohui Liu; liuguohui@hust.edu.cn

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Ferroptosis is an iron-dependent form of programmed cell death and an important type of biological catabolism. Through the action of divalent iron or ester oxygenase, ferroptosis can induce lipid peroxidation and cell death, regulating a variety of physiological processes. The role of ferroptosis in the modulation of bone homeostasis is a significant topic of interest. Herein, we review and discuss recent studies exploring the mechanisms and functions of ferroptosis in different bone-related cells, including mesenchymal stem cells, osteoblasts, osteoclasts, and osteocytes. The association between ferroptosis and disorders of bone homeostasis is also explored in this review. Overall, we aim to provide a detailed overview of ferroptosis, summarizing recent understanding on its role in regulation of bone physiology and bone disease pathogenesis.

1. Introduction

Ferroptosis is a form of cell death that was only recently defined by [1]. who proposed the concept in 2012 to describe a nonapoptotic type of cell death which is iron-dependent and is characterized by an accumulation of reactive oxygen species (ROS). Ferroptosis is closely related to a variety of metabolic disorders, tumors, and injuries [2–4]. During ferroptosis, the most susceptible lipids to peroxidation are polyunsaturated fatty acids (PUFAs).

In cell physiology, an increase of polyunsaturated fatty acids (PUFAs) on the cell membrane enhances the fluidity of the cell membrane, which indirectly increases the migratory ability of the cell [5]. Therefore, the increase in PUFAs is an important hallmark in the process of cell evolution. However, introduction of PUFAs also endangers cell survival. Hydrogen molecules produced during the dissociation of PUFAs can react with oxide and ferrous ions in the surrounding environment, resulting in the accumulation of peroxide and subsequent cell damage [6]. Normally, cells use PUFAs efficiently without causing cell damage by employing glutathione peroxidase 4 (GPX4) signaling which partially decreases the levels of PUFAs [7]. Mechanistically, GPX4 uses its catalytic activity to weaken the toxicity of lipid peroxides and maintain the homeostasis of lipid bilayer. Recent evidence identified the regulatory role of GPX4 and ferroptosis in multiple pathological processes. Currently, ferroptosis has attracted accumulative focus in studies on a wide range of diseases. Plenty of evidences have demonstrated that the pathological process of ferroptosis involves the excessive production of ROS, followed by abnormal activation of lipid

Table 1: The comparative characteristics among ferroptosis, apoptosis, and autophagy.

RCD	Ferroptosis	Apoptosis	Autophagy
Hallmarks	Mitochondrial crest disappeared; mitochondrial outer membrane rupture and shrinkage; mitochondria are deeply stained	Condensation and fragmentation of chromatin; nucleoli disappeared; nuclear pyknosis and fragmentation	Autophagy lysosome formation
Other characteristics	No nuclear rupture; cell membrane rupture	Cell shrinkage; the outflow of the cytoplasm and vacuolation of membrane	No changes in nuclear and cell membrane
Biomarkers	Upregulated: ROS, PTGS2; downregulated: NADPH	Cytochrome C releases caspase-activated intracellular calcium increases	Transformation from LC3-I into LC3-II
Positive regulators	Erastin, RSL3, RAS, Sorafenib, p53	P53, Bax, Bak, TGF-B, radiation	ATG family, Beclin1
Negative regulators	GPX4, FSP1, SLC7A11, NRF2, Ferrostatin-1, Liproxstatin-1, DFO	Bcl-2, Bcd-XL, Z-VAD-FMK	3-Methyladenine, Wortmannin, Spautin1

RCD: regulated cell death; PTGS2: prostaglandin endoperoxide synthase 2; FSP1: fibroblast-specific protein 1; NRF2: nuclear factor erythroid 2-related factor 2; DFO: desferrioxamine.

peroxidation in an iron-dependent manner, accompanied with a marked elevated uptake of PUFAs into the cellular membrane. The unique characteristics of ferroptosis make it complicated related to several biological processes.

Bone homeostasis is a physiological process regulated by bone related-stem cells, osteoblasts, osteoblasts, and osteocytes [8]. During bone remodeling, osteocytes, osteoblasts, and osteoclasts interact with one another in a paracrine manner and regulate angiogenesis in the bone marrow to maintain bone homeostasis [9]. Research has demonstrated the crucial role of ferroptosis in regulating the survival of bone-related cells and identified oxidative stress as an important factor in cell death [10, 11]. However, the exact mechanism of ferroptosis in bone homeostasis regulation remains largely unknown, and it is yet unclear whether ferroptosis is a driver or a passenger event in bone homeostasis.

Herein, we aim to review the recent literature on the subject to explore the underlying mechanisms of ferroptosis and its roles in different bone-related cells, including mesenchymal stem cells, osteoblasts, osteoclasts, and osteocytes. We summarize the recent findings on the role of ferroptosis in regulation of bone physiology and osteopathogenesis.

2. Ferroptosis

As previously mentioned, ferroptosis was first proposed in 2012 but was redefined as a mode of programmed cell death closely related to cell oxidative disturbance by the Nomenclature Committee on Cell Death in 2018 [12]. Compared to other classic forms of cell death, ferroptosis is characterized by the accumulation of iron-dependent lipid ROS. Ferroptosis occurs following a depletion of glutathione (GSH), subsequent decrease in the activity of glutathione peroxidase 4 (GPx4), and inhibition of lipid oxide metabolism as this is a GPX4-dependent reaction. Following this, divalent iron ions oxidize the lipid to produce ROS leading to ferroptosis [13].

Susceptibility to ferroptosis is closely related to multiple biological processes, including iron and PUFA metabolism and biosynthesis of GSH, phospholipid, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), and coenzyme Q10 [13]. It has also been linked to the pathological cell death seen in mammalian degenerative diseases, such as tumors, stroke, cerebral hemorrhage, traumatic brain injury, and renal failure [14, 15].

2.1. The Characteristics of Ferroptosis. During ferroptosis, a large number of iron ions are deposited in the dead cells, lipid peroxidation occurs intracellularly, ROS levels increase significantly, and proteins that regulate iron homeostasis and lipid peroxidation metabolism are altered [16]. Microscopically, the mitochondrial membrane shrinks, the mitochondrial crest decreases or disappears, and the outer membrane is broken, although the morphological changes of the nucleus are not as obvious (Table 1).

2.2. Underlying Mechanisms of Ferroptosis. Investigations on the regulation of ferroptosis have mainly focused on system Xc^- and GSH metabolism, regulation of GPX4 activity, and ROS production (Figure 1). System Xc^-, which comprises of SLC3A2 and SLC7A11 dimers, has been reported in various cells as a promising target for ferroptosis induction [17–19]. System Xc^- is embedded into the cell membrane and as an effective cystine/glutamate antiporter system regulates the transport of cysteine and glutamate [20]. Glutamate is transferred outside the cell, while simultaneously, cystine is imported into the cell where it participates in the generation of GSH and thereby prevents ferroptosis [21]. A recent study reported that IFN-γ was capable of suppressing the expression of SLC3A2 and SLC7A11 via activation of JAK/STAT signaling, and repression of system Xc^- could induce ferroptosis in hepatocellular carcinoma cells [22]. Similarly, as a tumor suppressor gene, p53 was demonstrated to inhibit cystine uptake by downregulating the expression of SLC7A11, which could decrease the activity of GPX4 and reduce the antioxidant ability of the cells ultimately inducing ferroptosis [23].

Furthermore, GPX4 is considered a crucial molecule in the regulation of ferroptosis [24]. The basis of ferroptosis is

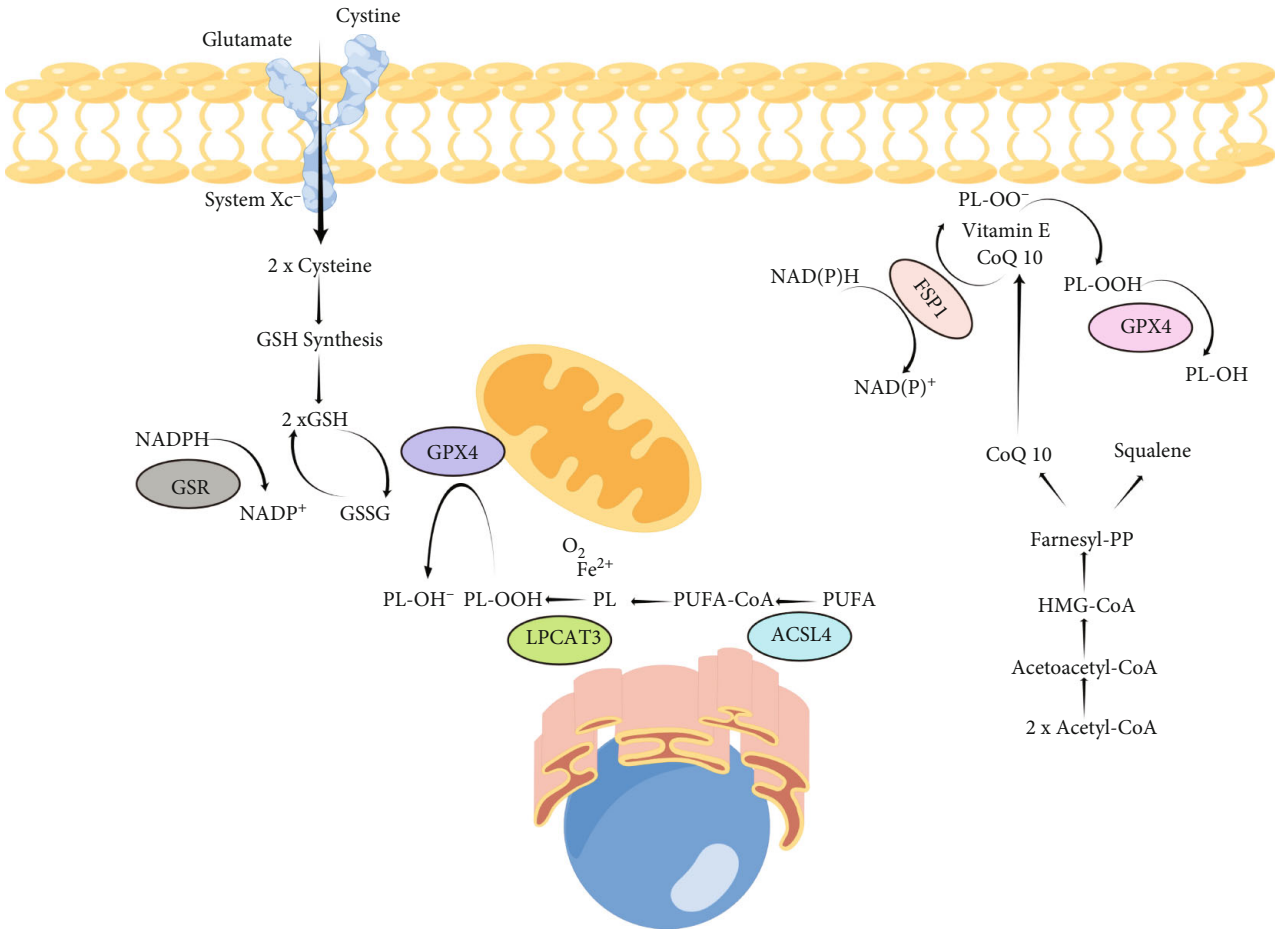

Figure 1: The molecular mechanism of ferroptosis.

the presence of free iron in the cells. The Fenton reaction between iron ions and ROS leads to peroxidation of PUFAs and formation of lipid peroxides, resulting in damage to the cell membrane [25]. GPX4 is able to ameliorate the toxicity of lipid peroxides via its catalytic activity and maintain the homeostasis of the lipid bilayer. Prior studies have shown that RSL3, an inhibitor of GPX4, can covalently bind to GPX4 and inactivate it, ultimately leading to the accumulation of intracellular peroxide and induction of ferroptosis [26].

In addition, the ROS-mediated pathway is a critical mechanism of ferroptosis. As induction of ferroptosis leads to the increase of intracellular lipid ROS, theoretically, lipid antioxidants may be promising antiferroptosis agents [27]. Mitochondria, an organelle with abundant iron and ROS production, are considered to be an important location of the occurrence of ferroptosis.

2.3. Mitochondrial Dysfunction Regulates Ferroptosis. Given the important role of mitochondria in ROS generation, their function is critical in ferroptosis [28]. Prior research revealed that complete inhibition of mitochondrial function could significantly decrease cell sensitivity to ferroptosis under cysteine-deprivation conditions [29]. Furthermore, Gaschler et al. [30] reported that partially decreased functioning of mitochondria could restore cell sensitivity to ferroptosis, findings which highlight mitochondria's significant function in initiating ferroptosis. Suppression of the tricarboxylic acid (TCA) cycle and electron transport chain (ETC) was also demonstrated to inhibit ferroptosis, which is consistent with the role of mitochondria in ROS generation [29, 30]. Several enzymes in the TCA cycle are critical for inducing ferroptosis [31]. For instance, a recent study showed that deprivation of fumarate hydratase in renal cancer cells could increase cell tolerance to ferroptosis [32]. Moreover, disruption of the TCA cycle was capable of suppressing lipid peroxidation and ferroptosis [33]. Consistent with the significant role of this process in mediating ferroptosis, suppression of the ETC was found to inhibit ROS accumulation and the induction of ferroptosis in response to either cysteine deprivation or erastin (a ferroptosis inducer) treatment [29].

3. Ferroptosis and Bone Homeostasis

Homeostasis is a complicated balance that is crucial for cells to maintain their normal physiological functions [30]. A tight balance between energy input and consumption is important for cell homeostasis. During metabolism, cells continuously consume energy and nutrients, while producing new energy and nutrients [31]. Similarly, our skeletal

system has a continuous remodeling cycle, and an appropriate balance between anabolism and catabolism is needed to maintain the strength and healthy microstructure of bone tissue [32]. Bone remodeling is accomplished through the coordinated efforts of four key cells: bone marrow mesenchymal stem cells (MSCs) which are the source of osteoblasts (OBs) and exert regulatory functions throughout remodeling; OBs are located on the bone surface and secrete bone matrix; matrix-embedded OBs further differentiate into persistent osteocytes (OCT), which form a mechanosensory network in bone and play a crucial role in paracrine signaling. At the same time, osteoclasts (OC) continuously degrade and absorb the surrounding bone base [33]. The dynamic balance between bone formation and bone resorption is continuously coordinated. As ferroptosis is an important mode of regulated cell death, its relationship with skeletal cells, including MSCs, OBs, OCs, and OCTs, has attracted attention in recent decades [34].

3.1. Ferroptosis and the MSCs. Recent research in the study of bone tissue repair and regeneration has paid particular attention to MSCs. MSCs have the potential of multidirectional differentiation with low immunogenicity and wide availability. They can migrate to damaged tissues and organs to reconstruct these through direct differentiation or secretion of exosomes, growth factors, and cytokines [35, 36]. Furthermore, the regulatory role of MSCs in ameliorating cell ferroptosis has been well-documented [37]. For example, it was recently shown that MSCs are capable of inhibiting the production of lipid peroxidation and alleviating ferroptosis both *in vitro* and *in vivo*. The authors also demonstrated that MSC-derived exosomes are involved in the underlying mechanisms of the effect of MSCs on ferroptosis, which could significantly downregulate the expression of prostaglandin-endoperoxide synthase 2 and promote SLC7A11 expression [38]. Similarly, the suppressive effect of MSCs on ferroptosis was seen in neuronal cells [39]. In an acute spinal cord injury mouse model, researchers demonstrated that MSCs and their exosomes could ameliorate spinal cord injury through promotion of the expression of ferroptosis inhibitor (FSP1) [39]. In addition to the discovery of the antiferroptotic effect of MSCs, the underlying mechanism of ferroptosis in MSCs was also investigated. It is well-documented that NOP2/Sun RNA methyltransferase 5 (NSUN5) posttranscriptionally can mediate ferroptosis in MSCs through RNA methylation [40]. A recent study further found that NSUN5 is downregulated in erastin-induced ferroptosis in MSCs, while NSUN5 is capable of suppressing ferritin heavy chain/light-chain (FTH1/FTL) activity. In the NSUN5 depletion experiments, they found an accumulation of intracellular iron and a marked decrease of GPX4, suggesting that the NSUN5-FTH1/FTL pathway mediates ferroptosis in MSCs and that therapeutic targeting of components of this pathway may promote resistance to ferroptosis and improve the survival of MSCs [40].

3.2. Ferroptosis and OBs. The integrity of bone is maintained through an appropriate balance between osteogenic and osteoclastic activities, and the bone remodeling process is a continuous cycle. OBs are mainly involved in bone reconstruction, including formation, mineralization, and construction of osteocytes [41]. A variety of studies have focused on the potential mechanisms and agents regulating OB ferroptosis [42]. Advanced glycation end products were recently found to induce OB ferroptosis and promote osteoporosis [43]. Inversely, melatonin, a hormone secreted by the pineal gland, was shown to ameliorate OB ferroptosis and enhance the osteogenic capacity of OB via activation of Nrf-2/HO-1 signaling [44]. Mechanistically, mitochondrial ferritin (FtMt) was reported to exert a critical role in regulating cell ferroptosis via storing iron ions and intercepting toxic ferrous ions in mitochondria [45]. The researchers found that activation of FtMt could ameliorate OB ferroptosis while inhibition of FtMt could induce mitophagy through ROS/PINK1/Parkin signaling [45]. Moreover, increased ferroptosis in OBs could be seen after activating mitophagy, with the findings suggesting that FtMt can effectively suppress ferroptosis in OBs [45]. Interestingly, exosomes, extracellular vesicles containing active regulatory factors, have been shown to participate in the regulation of ferroptosis in OB. For example, one recent study reported that vascular endothelial cells could effectively prevent osteoblastic ferroptosis through exosome release which could further suppress ferritinophagy and limit ferroptosis of OBs [46]. Similarly, using an osteoporotic murine model, it was reported that exosomes from endothelial progenitor cells could inhibit steroid-induced osteoporosis through suppression of the ferroptotic pathway [47].

3.3. Ferroptosis and OCs. Iron ions is capable to induce OC differentiation and bone resorption through the production of ROS [48, 49]. Zoledronic acid (ZA), a bisphosphonate, has been reported to inhibit OC growth via induction of ferroptosis of the OC [49]. The role of ZA in regulating osteoclast function was evaluated using a RANKL-induced cell model, which indicated that ZA treatment suppressed the cell viability of osteoclasts and facilitated osteoclast ferroptosis with an increase in iron ions and ROS and decrease in the GPX4 and GSH level [49]. Similarly, ferroptosis was reported involved in OC function during RANKL-induced differentiation and is induced by iron starvation response and ferritin phagocytosis [50]. Mechanically, subsequent RANKL stimulation can lead to iron droop due to iron starvation response (increased transferrin receptor 1 and decreased ferritin) under normoxic but not hypoxic conditions, due to downregulation of aconitase activity [50]. Based on these results, it can be assumed that ferroptosis of OCs can limit bone resorption, while inducing ferroptosis in OCs could be an alternative treatment of disorders of bone formation.

3.4. Ferroptosis and OCTs. OCTs, the most prevalent cells in mineralized bone tissue, communicate with other bone cells, such as OBs and OCs, via the lacunar-canalicular system and through various secreted hormones [51]. Decreased activity and death of OCTs induced by internal and external factors can lead to bone loss and destruction of bone microstructure. Therefore, effective promotion of OCT survival is a

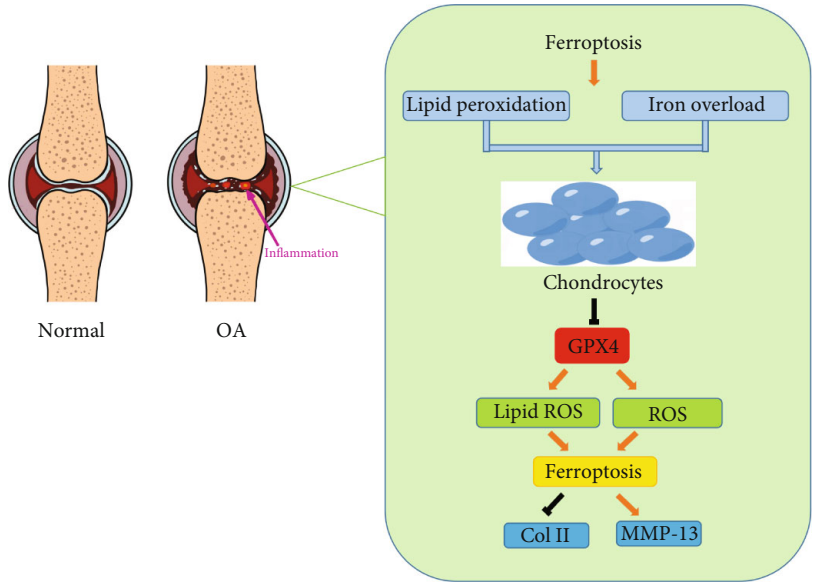

Figure 2: The potential mechanism of ferroptotic chondrocytes.

promising therapeutic strategy for maintenance of bone homeostasis. It has been reported that ferroptosis is an important form of OCT death, which can be reversed by targeting the inhibition of ferroptosis signaling pathways [52]. Yang et al. [53] found that a hyperglycemic microenvironment is capable of promoting lipid peroxidation and iron overload, thereby inducing osteocyte ferroptosis. Furthermore, RNA sequencing results indicated that heme oxygenase-1 (HO-1) is overexpressed in ferroptotic osteocytes, suggesting that HO-1 is essential for osteocyte ferroptosis. Similarly, a recent study reported that dexamethasone could notably induce ferroptosis in MC3T3-E1 cells (a type of OCT precursor cell) via downregulation of the p53/SLC7A11/GPX4 signaling pathway, providing a potential mechanism for the effect of ferroptosis on osteocytes in steroid- (glucocorticoid) induced osteonecrosis of the femoral head [54]. These findings highlight a potential therapeutic target for the treatment of skeletal disorders.

4. Ferroptosis and Bone Degenerative Disorders

Ferroptosis differs from apoptosis, autophagy, necrosis, and pyrodeath, in that it mainly involves iron metabolism and lipid peroxidation. Ferroptosis plays an important role in malignant tumors, cardiovascular diseases, and neural system diseases [55, 56]. Iron overload is closely related to cellular ferroptosis, and iron overload and lipid peroxide accumulation jointly mediate bone destruction, ultimately leading to bone disorders.

4.1. Ferroptosis and Osteoporosis. Osteoporosis is a systemic bone disease characterized by a reduction in bone mass and a degeneration of the fibrous structure of bone tissue, which results in increased bone brittleness and risk of fracture [57]. Its pathological features include the following [58]: (1) decreased bone mass, including a reduction in the proportion of bone minerals and other substrates; (2) degeneration of bone microstructure, caused by absorption and imbalance of bone tissue homeostasis, manifesting as destruction, deformation, and fracture of bone trabecular structure; and (3) increased bone brittleness and decreased bone strength, increased fracture deformation, decreased load bearing force, and more frequent microfracture or complete fracturing. Iron is a strong oxidant that can promote the production of ROS radicals, and iron metabolism can directly or indirectly affect the occurrence and development of type 2 diabetes [59, 60]. Ferroptosis results in the production of abundant ROS through the Fenton reaction, inducing accumulation of lipid peroxides and cell damage [61]. It was well-documented that hyperglycemia can induce ferroptosis in the bone tissue of an osteoporotic rat model by production of ROS/lipid peroxidation. Melatonin was shown to ameliorate the level of ferroptosis through activation of Nrf2/HO-1 signaling and promotion of the osteogenic differentiation of MC3T3-E1 cells [44]. Similarly, in a murine model of diabetic osteoporosis (DOP), the researchers verified the important role of ferroptosis in DOP-induced OCT death. Mechanistically, activation of Nrf2/HO-1 signaling could lead to lipid peroxidation and cell ferroptosis, suggesting that targeting inhibition of OCT ferroptosis may be a potential therapeutic strategy for DOP treatment [53].

Furthermore, the relationship between ferroptosis and glucocorticoid-induced osteoporosis (GIOP) has been well investigated [62]. For example, a recent study [47] reported that high-dose dexamethasone (10 μM) can induce ferroptosis of OB by inhibiting the expression of GPX4 and system Xc^-. To investigate the underlying mechanisms, extracellular vesicles were extracted from bone marrow-derived endothelial progenitor cells (EPC-EVs), which were seen to suppress ferroptosis by restoring the activity of GPX4 and system Xc^-. Significantly, EPC-EVs were capable of reversing dexamethasone-induced changes in cysteine and oxidative damage markers and improved skeletal parameters in mice. These results suggest that EPC-EVs reverse murine GIOP through inhibition of OB ferroptosis.

4.2. Ferroptosis and Osteoarthritis (OA). OA is a degenerative disease characterized by the pathological alteration of the function and morphology of an entire joint, as well as articular cartilage destruction and damage to other joint components [63–66]. Generally, OA occurs due to chronic heavy loading and biomechanical damage; however, pathological progress at the molecular level has also been proposed in the development of OA. Therefore, maintaining chondrocytes in a healthy state is considered to be an effective strategy for preserving the integrity of the entire cartilage [67–70]. It can be, therefore, assumed that ferroptosis may be involved in the progression of OA. In a recent study [71], researchers used interleukin-1 beta (IL-1β) to construct an *in vitro* iron-overload model. The authors found that IL-1β could induce both ROS and lipid ROS accumulations and saw ferroptosis-related protein expression changes in the chondrocytes. Furthermore, increased MMP13 expression and decreased collagen II expression were seen in the ferroptotic chondrocytes (Figure 2). In a murine OA model, intra-articular injection of a ferroptosis inhibitor was seen to prevent OA progression. These findings highlight the contribution of ferroptosis in chondrocytes to the progression of OA. Studies have also been conducted to identify a feasible treatment for chondrocyte degeneration with a focus on cell ferroptosis [72]. Deferoxamine (DFO) [73] and D-mannose were recently demonstrated to alleviate OA progression by inhibiting of chondrocyte ferroptosis. DFO was found to both effectively ameliorate chondrocyte ferroptosis and induce activation of the Nrf2 antioxidant system, which is crucial for chondrocyte protection [73]. The efficacy of injection of DFO in OA mice was also demonstrated *in vivo* [73]. Similarly, Zhou et al. [74] investigated whether D-mannose mediates chondrocyte ferroptosis during OA cartilage degeneration *in vitro* and *in vivo*. They found that D-mannose could exert a chondroprotective effect by attenuating the sensitivity of chondrocytes to ferroptosis and could alleviate OA progression. Furthermore, HIF-2α was identified as a central mediator in the D-mannose-induced resistance of chondrocytes to ferroptosis. These findings provide potential therapeutic strategies for ferroptosis-related bone diseases.

5. Conclusion

During the past decades, there has been an accumulative research focus on the relationship between ferroptosis and diseases [75, 76]. The significance of ferroptosis in cell survival and differentiation is widely accepted, and its regulatory role in the modulation and treatment of diverse disease has been gradually uncovered [77, 78]. However, there are still some academic problems yet to be resolved. For example, the relationship between ferroptosis and the other forms of regulated cell death in the regulation of skeletal disorders should be further revealed. Furthermore, the detailed molecular mechanisms that activate ferroptosis are still unascertained. In addition, plenty of emerging evidences have demonstrated that exosomes are involved in the modulation of ferroptosis and skeletal disease [79, 80]. In-depth knowledge of this exosome-mediated effect should be achieved by performing more researches regarding the crosstalk between exosome and ferroptosis.

The recent continuous efforts in the research of ferroptosis have shed light on the interaction between ferroptosis and bone homeostasis. Technical limitations currently restrict the in-depth understanding of the mechanisms underlying ferroptotic regulation. Specifically, lack of an effective and specific ferroptotic blocker precludes the observation of the effect of blockade on the physiological functions of ferroptosis in *in vivo* models. Furthermore, lack of defined and specific ferroptotic signaling pathways or biomarkers hinders the verification of ferroptosis in physiological or pathological conditions. In addition, given the limitations of existing experimental techniques, we do not yet have a visual reporting method of *in vivo* ferroptosis detection. In future studies of ferroptosis, research should focus on in-depth study of the molecular mechanisms underlying ferroptosis and screening and identifying specific signaling pathways. Furthermore, efforts should be invested in developing a feasible detectable tool for measuring ferroptosis *in vivo*. Finally, the regulatory role of ferroptosis in the process of bone aging should be elucidated.

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 they have no conflicts of interest.

Authors' Contributions

YX, BM, and GL conceived the ideas. YX, LC, ACP, and ZL wrote the manuscript. ACP edited the manuscript. YH, WZ, YS, FC, GL, GD, and RZ participated in the discussions. Yuan Xiong, Ze Lin, and Lang Chen contributed equally to this work.

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Review Article

Role of Primary Cilia in Skeletal Disorders

Xinhua Li,[1] **Song Guo**,[1] **Yang Su**,[2] **Jiawei Lu**,[3] **Donghua Hang**,[1] **Shao Cao**,[1] **Qiang Fu**,[1] **and Ziqing Li**[4,5]

[1]*Department of Orthopedics, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, China*
[2]*Department of Joint Surgery, Shandong Provincial Hospital, Shandong University, Jinan 250012, China*
[3]*Department of Spinal Surgery, Shanghai East Hospital, Shanghai Tongji University School of Medicine, Shanghai 200120, China*
[4]*Department of Joint Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan 250021, China*
[5]*Orthopaedic Research Laboratory, Medical Science and Technology Innovation Center, Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan 250117, China*

Correspondence should be addressed to Qiang Fu; johson.f@163.com and Ziqing Li; liziqing@sdfmu.edu.cn

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Primary cilia are highly conserved microtubule-based organelles that project from the cell surface into the extracellular environment and play important roles in mechanosensation, mechanotransduction, polarity maintenance, and cell behaviors during organ development and pathological changes. Intraflagellar transport (IFT) proteins are essential for cilium formation and function. The skeletal system consists of bones and connective tissue, including cartilage, tendons, and ligaments, providing support, stability, and movement to the body. Great progress has been achieved in primary cilia and skeletal disorders in recent decades. Increasing evidence suggests that cells with cilium defects in the skeletal system can cause numerous human diseases. Moreover, specific deletion of ciliary proteins in skeletal tissues with different Cre mice resulted in diverse malformations, suggesting that primary cilia are involved in the development of skeletal diseases. In addition, the intact of primary cilium is essential to osteogenic/chondrogenic induction of mesenchymal stem cells, regarded as a promising target for clinical intervention for skeletal disorders. In this review, we summarized the role of primary cilia and ciliary proteins in the pathogenesis of skeletal diseases, including osteoporosis, bone/cartilage tumor, osteoarthritis, intervertebral disc degeneration, spine scoliosis, and other cilium-related skeletal diseases, and highlighted their promising treatment methods, including using mesenchymal stem cells. Our review tries to present evidence for primary cilium as a promising target for clinical intervention for skeletal diseases.

1. Introduction

Primary cilia are highly conserved microtubule-based organelles that project from the cells' surface into the extracellular environment and play important roles in mechanosensation, mechanotransduction, and polarity maintenance during organ development and pathological changes, including in the skeletal system [1, 2]. The assembly and function of cilia require effective intraflagellar transport (IFT) in the cilium, which is a bidirectional transport operated by IFT protein complexes and IFT motors. IFT protein complexes are divided into complex A and complex B [3]. In primary cilia, membrane cargos are trafficked in vesicles to the ciliary base by the Bardet-Biedl syndrome (BBSome) coat complex [4]. The IFT gene or BBS gene mutation can cause cilium defects or loss. Primary cilia have a complex function including transducing Hedgehog signaling and sense and transduce chemical or mechanical signal. The role of cilia in Hedgehog signaling transduction was found by Huangfu et al. in 2003, and the process has been well established [5]. In the absence of ligand, Patched (Ptch) prevents translocation of Smoothened (Smo) to the plasma membrane. A microtubule-associated complex promotes the processing of Gli into its repressor form. Upon activation of the pathway, Smo moves

to the plasma membrane, and the Sufu-Gli complex associates with the carboxy-terminal tail of SMO, resulting in the release of Gli and promotion of the processing of Gli into its activated form [6].

The skeletal system consists of bones, cartilage, intervertebral disc (IVD), tendons, and ligaments, providing support, stability, and movement to the body. Skeletal systems are exposed to various mechanical loads and function as a major system for the mechanical transduction in our body. Primary cilia were regarded as a chemical or mechanosensor and signaling pathway transduction center. Therefore, it is believed that cilia have a critical role in skeletal function. In the last several years, emerging studies reported that cells with cilium defects in skeletal systems can cause many human diseases, including osteoarthritis (OA) and intervertebral disc degeneration (IVDD), tendinopathy, Jeune's syndrome, and spinal scoliosis [7–11]. In addition, the primary cilium is well known to play an important role in osteogenic/chondrogenic induction of mesenchymal stem cells (MSCs), regarded as a promising target for clinical intervention for skeletal disorders. New approaches to treat osteoporosis, OA, and other skeletal disorders have focused on promoting bone or cartilage formation through the targeting of osteoblasts/chondrocyte and their progenitors or MSCs. Here, we reviewed available literatures on primary cilia and their role in skeletal disorders and their promising treatment methods, including MSCs.

2. Primary Cilia and Ciliary Proteins in Skeletal Diseases

2.1. Primary Cilia and Osteoporosis and Fracture Healing. Osteoporosis is one of the most prevalent chronic skeletal pathologic diseases characterized by decreased bone mass, placing an enormous economic burden on patients and payors all over the world [12]. As an exquisitely mechanosensitive organ, mechanical stimulation deficiency has been regarded as a leading cause of osteoporosis. Primary cilia are sensory organelles that play an important role in translating extracellular chemical and mechanical cues into cellular responses and are believed to be closely related to bone development and osteoporosis. The essential role of primary cilia on bone development and patterning has been well established [1, 13, 14] (Table 1). Also, knockout of many cilium-related genes leading to the cilium defects was reported to cause mouse long bone or vertebral osteoporosis phenotype, including IFT80, IFT88, Kinesin family member 3A (Kif3a), Evc, Pkd1, and IFT40 [11, 15–21].

New methods to treat osteoporosis focused on promoting osteogenic induction of MSCs, and the primary cilia were reported to be essential for MSCs' osteogenic differentiation [22]. Corrigan et al. [23] found that LiCl and fenoldopam can be utilized to enhance ciliogenesis in MSCs and fenoldopam is a viable ciliotherapeutic option to enhance MSCs' osteogenesis and potential to treat osteoporosis. However, how the cilium changed in osteoblasts or osteocytes during osteoporosis is largely unknown. Further study to identify the relationship between primary cilia and osteoporosis needed to be investigated.

Fracture healing is a complex biological process that shares some similarity feature with the bone development. Recently, Liu et al. [24] found that conditional deletion of IFT80 in chondrocytes utilizing tamoxifen-inducible Col2-CreER mice resulted in low-density/porous woven bony tissue compared to control during fracture healing. Mechanistically, IFT80 deletion can downregulate the TGF-β signaling pathway by inhibiting the expression of TGF-βI and TGF-βR and phosphorylation of Smad2/3 in the fracture callus. Chinipardaz et al. [25] reported that loss of cilia caused by diabetes in osteoblasts resulted in defective diabetic fracture healing by using in a streptozotocin-induced diabetes and Osx-cre;IFT80$^{fl/fl}$ mouse model. All these demonstrated that cilia are important in bone fracture healing.

2.2. Primary Cilia and Bone or Cartilage Tumors. Bone or cartilage tumors are one of the most common human primary bone lesions, and they range from benign lesions, such as enchondromas and osteochondromas, to malignant chondrosarcoma [26]. Enchondromas and osteochondromas are the most common benign bone tumor, and they are always developing during periods of bone growth in a location adjacent to the growth plate [26, 27]. Enchondromas occur within the metaphyseal portion of bone. Osteochondromas manifest as outgrowths of bone and cartilage from the metaphyseal region of long bones, with a cartilage cap on. The development of bone or cartilage tumors is always combined with constitutively active hedgehog (Hh) signaling. The primary cilium is the center for the Hh signaling transduction; thus, the relationship between cilia and osteochondromas and enchondromas has been investigated. In osteochondroma, the primary cilium incidence was normal, but the cilium orientation was dramatically disrupted compared with control [28]. Cilium organization is essential for cells' polarity, and the disorganized cilium orientation in most cells of osteochondromas may contribute to the loss of cell polarity and arrangement in the growth plate [29]. However, the cilium incidence in enchondroma is reported to vary in different studies [28]. Ho et al. [30] reported that only 13.4% of cells are ciliated in enchondroma tissues, which significantly decreased compared with control articular cartilage (Figure 1). Recently, we found that the cilium incidence and cilium length were comparable between human enchondroma cells and control articular chondrocytes, but the cilium orientation largely alters [31]. The different sample resources may contribute to variation in different samples, and the cilium features in more human enchondroma samples are needed to be identified in the future. Chondrosarcoma is a cartilaginous origin malignant tumor with aggressive behavior. In human and mouse chondrosarcomas, the cilium incidence of neoplastic chondrocytes is dramatically lost compared with normal articular cartilage [30]. Parts of chondrosarcoma are thought to originate from benign tumors when combined with P53 mutation [26]. The dramatic decreased cilium incidence from osteochondromas to chondrosarcoma transition suggested that the percentage of ciliated cells can serve as a useful marker to distinguish benign and malignant tumors.

Table 1: The role of primary cilia in bone development illustrated by the conditional knock out mouse model.

Gene	Function
IFT20	Col1-CreERT;IFT20$^{fl/fl}$ and Osx-Cre;IFT20$^{fl/fl}$ mice exhibit reduced bone mass and strength. Deletion of IFT20 impairs osteoblast polarity and cell alignment via ceramide-PKCζ-β-catenin signaling [96]
IFT140	Osx-Cre;IFT140$^{fl/fl}$ mice exhibited dwarf phenotypes, such as short bone length, less bone mass, and decreased bone mineral apposition rate [21]
IFT80	Osx-Cre;IFT80$^{fl/fl}$ mice show reduced bone mass with impaired osteoblast differentiation; IFT80 is required for osteoblast differentiation by balancing between canonical and noncanonical Hedgehog pathways [11]
KIF3a	Osx-Cre;Kif3a$^{fl/fl}$ mice display an osteopenia phenotype with impaired osteoblast function. Kif3a deletion in osteoblast impairs osteoblast-mediated bone formation through multiple pathways including intracellular calcium, hedgehog, and Wnt signaling [16] Col1-Cre;Kif3a$^{fl/fl}$ mice have normal bone development but reduced bone formation in response to a cyclic ulnar loading [97]
PKD	Osx-Cre;Pkd1$^{flox/m1Bei}$ mice show reduced bone mass, mineral apposition rates, increased adipogenesis in bone marrow, and impaired osteoblast differentiation [19]

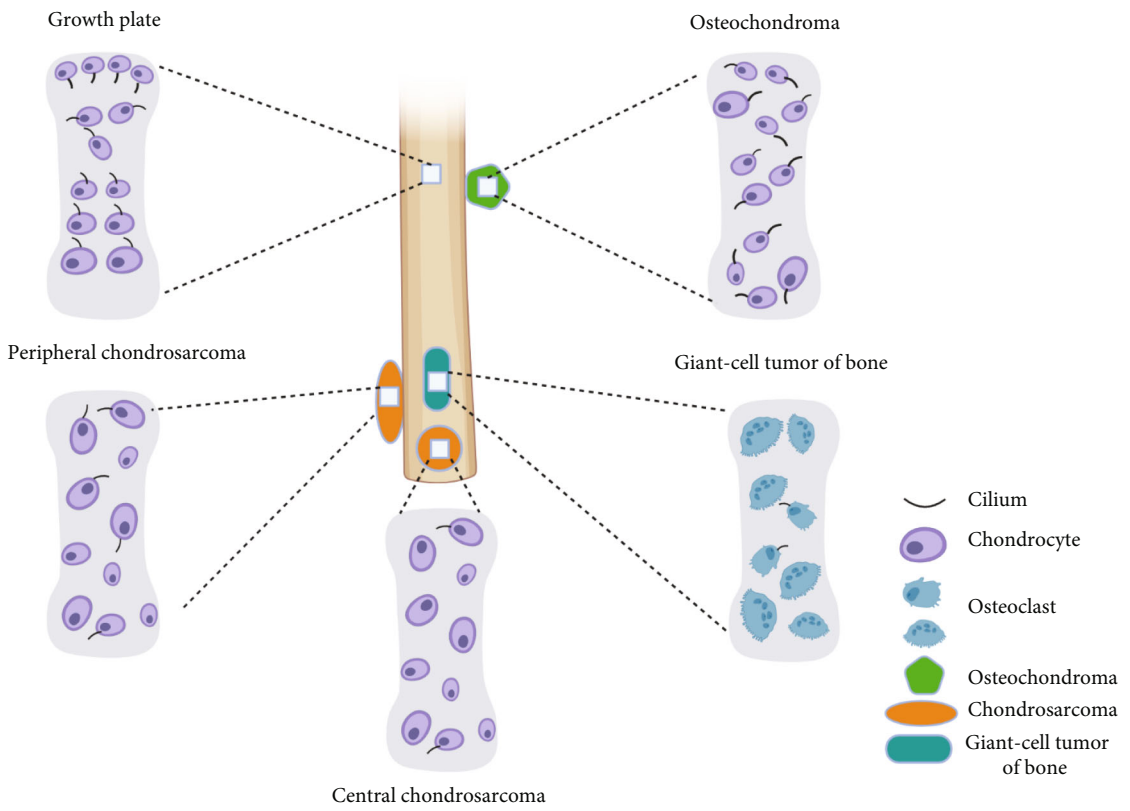

Figure 1: Schematic representation shows the cilium feature in bone or cartilage tumors. In both the proliferative and hypertrophic zones of the normal growth plate, the cilium is well orientated as shown in each layer. However, in osteochondroma, chondrocyte arrangement and cilia orientation are dramatically disorganized. In human malignant chondrosarcomas, cilium incidence is reduced and cilium orientation is disorganized. In the mouse peripheral chondrosarcoma, primary cilium is dramatically reduced and cilium orientation is disorganized. Giant cell tumor of bone is composed of mononuclear stromal cells and numerous macrophage giant cells, but only mononuclear stromal cells of giant cell tumor of bone present primary cilia.

Hedgehog (Hh) concentration distribution gradient is essential for normal chondrocyte proliferation and differentiation. However, the Hh gradient is disrupted and showed a homogeneous pattern in enchondroma or osteochondroma [31]. Activated Hh signaling in the growth plate (Col2α1-Gli2 overexpressed mouse) leads to enchondroma in mice. Similar to the Col2α1-Gli2 mouse, IFT88 partial mutant also developed enchondroma around the growth plate. Interestingly, activated Hh signaling (Gli2-overexpressed) in the IFT88 deficiency mouse can cause much more enchondromas. The disruption of cilia in Gli2-overexpressed mouse results in much more enchondromas, suggesting that cilia can inhibit Hh signaling activation under these conditions [30]. Some studies [32, 33] suggested that Hh signaling is essential for bone tumor growth and process; loss of primary cilium-disrupted Hh signaling can inhibit tumor growth or process. However, we found that Indian hedgehog (Ihh) ablation in aggrecan-positive progenitors produced

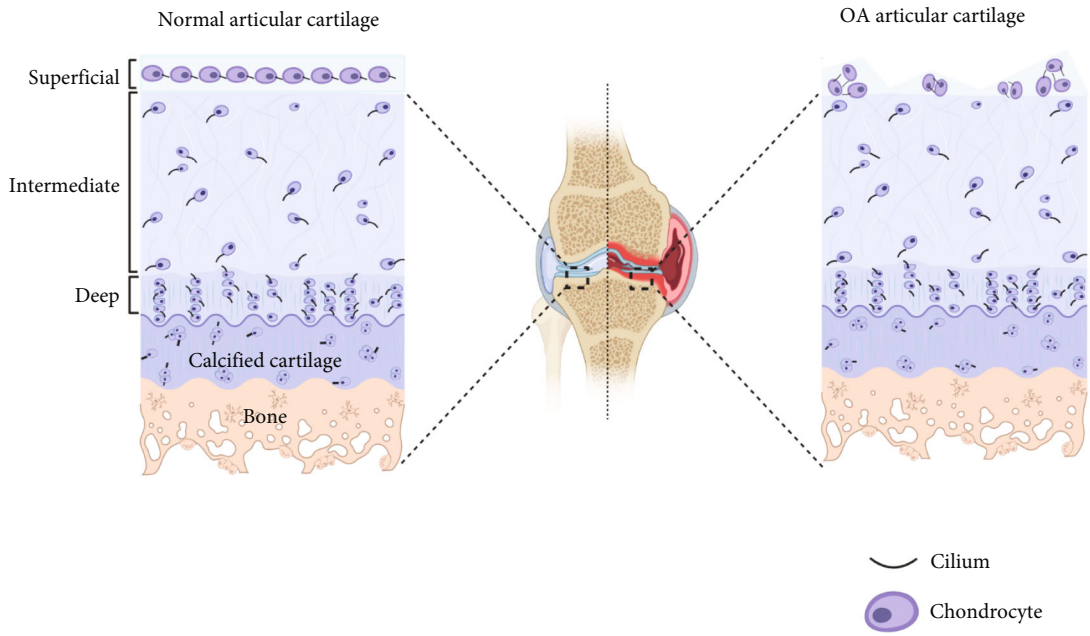

Figure 2: Cilium in normal and osteoarthritis (OA) articular cartilage tissues. The normal articular cartilage can be divided into superficial, intermediate, and deep zones as shown in figures. In the superficial zone of normal articular cartilage (left), the chondrocytes are ellipsoid. Both chondrocyte and cilia are parallel to the surface of articular cartilage. In intermediate and deep zones, the chondrocytes are irregular, but the cilium orientation is on the medial or lateral cell membranes along the longitudinal axis parallel to the chondrocyte. However, the articular surface is eroding in human OA tissue, and the cilium incidence and length significantly increased compared with normal human articular cartilage [40]. Moreover, the cilia are oriented parallel to the long axis of cells at the articulating surface in normal articular cartilage, but it is oriented to the center of abnormal cell clusters in osteoarthritic cells.

enchondroma-like tissues near the growth plates in mice, and smoothen agonists can significantly reduce the enchondroma incidence in Ihh-knockout mouse [31]. Consistently, the dual and opposing roles of primary cilia and Hh signaling were also found in medulloblastoma development [34]. How cilia and Hh signaling are involved in bone tumor development and progress needs to be investigated furtherly.

Ciliogenesis and elongation processes require the coordination of microtubule assembly and protein modification. Histone deacetylase 6 (HDAC6), as a special member of the HDAC family, plays a vital role in microtubule deacetylation [35]. Xiang et al. [35] reported that a significant decrease in cilium expression and abnormal expression of HDAC6 existed in human chondrosarcoma tissues, and targeting inhibition of HDAC6 could significantly suppress chondrosarcoma cell proliferation and invasion. The potential mechanism may affect ciliogenesis via the Aurora A-HDAC6 cascade. Although these in vitro data on the therapeutic effect of HDAC inhibition on chondrosarcoma are promising, the data from the clinical trial are discouraging in patients [36]. Whether HDAC inhibition or other drugs targeting cilia or cilium-related signaling are effective in the treatment of chondrosarcoma remains to be demonstrated in the future [37].

Giant cell tumor of bone, which usually appears in long bone epiphysis in young adults, is a locally aggressive primary bone neoplasm composed of proliferative mononuclear stromal cells, numerous reactive macrophages, and large osteoclast-like multinucleated giant cells. Castiella et al. [38] found that mononuclear stromal cells of giant cell tumor of bone present primary cilia, and the Hh signaling pathway is activated in these cells. They speculated that primary cilia may play an important role in giant cell tumor of bone tumorigenesis and could be used as a potential therapeutic target in the future.

2.3. Primary Cilia and OA. OA is one of the most prevalent joint diseases of advanced age and is a leading cause of disability worldwide. OA patients usually suffer from many annoying complications that negatively influence their quality of life. In pathophysiology, OA is characterized by the degeneration of articular cartilage and elevated chondrocyte mortality [39]. Abnormal mechanical overload has been found to be one of the major contributions to the onset and progression of OA. Primary cilia, which have been found crucial in biomechanical signaling transduction, are linked to OA by many studies in the last several years. Primary cilia were found present on both normal articular cartilage and OA tissue, and the cilium incidence and length significantly increased in the eroding articulating surface of human OA compared with normal human articular cartilage [40] (Figure 2). Moreover, the cilia are oriented parallel to the long axis of cells at the articulating surface in normal articular cartilage, but they are oriented to the center of abnormal cell clusters in osteoarthritic cells [40]. Alkaptonuria (AKU) is an inherited disease resulting from a deficiency of the enzyme homogentisate 1,2-dioxygenase which is characterized by severe cartilage degeneration, similar to that observed in OA. However, Thorpe et al. [41] found that the cilium length is dramatically decreased in AKU articular

Table 2: The role of primary cilia in cartilage development illustrated by the conditional knock out mouse model.

Gene	Function
IFT20	Col2-cre;Ift20$^{fl/fl}$ has normal limb development, but Prx-cre;Ift20$^{fl/fl}$ mouse shows four limb development defects. Deletion of Ift20 increased Fgf18 expression in the perichondrium that sustained Sox9 expression, thus preventing endochondral ossification [98]
IFT80	Deletion of IFT80 in the embryonic stage (injected tamoxifen at embryonic day 14.5 in Col2-creERT;IFT80 mouse) shows shortened cartilage and limbs at birth; deletion of IFT80 in the postnatal stage (injected tamoxifen at postnatal day 4 in Col2-creERT;IFT80 mouse) causes reduced growth plate length; loss of IFT80 blocks chondrocyte differentiation by disruption of ciliogenesis and alteration of Hh and Wnt signaling transduction, which in turn alters epiphyseal and articular cartilage formation [99]
IFT88	Col2-Cre;Ift88$^{fl/fl}$ mice display disorganized columnar structure and early loss of growth plate; Ift88 regulates the expression of Sfrp5 and Wnt signaling pathways in the growth plate via regulation of Ihh signaling [9] Aggrecan-CreERT;Ift88$^{fl/fl}$ mice have a thinner articular cartilage thickness in the middle of tibia at 33 weeks old [43]
KIF3a	Col2α1-Cre;Kif3a$^{fl/fl}$ mice show postnatal dwarfism with a disorganized growth plate and altered chondrocyte orientation; deletion of Kif3a inhibits cell proliferation but accelerates hypertrophic differentiation, leading to the premature closure of the growth plate [100]
KIF5b	Col2α1-Cre;Kif5b$^{fl/fl}$ mice were smaller in stature owing to shortened spine vertebrae and long bones; mutant mice characterized by disorganized columnar structure in the growth plates; Kif5b mutation can cause incomplete cell rotation, proliferation, and differentiation disruption and results in a disorganized growth plate [101]

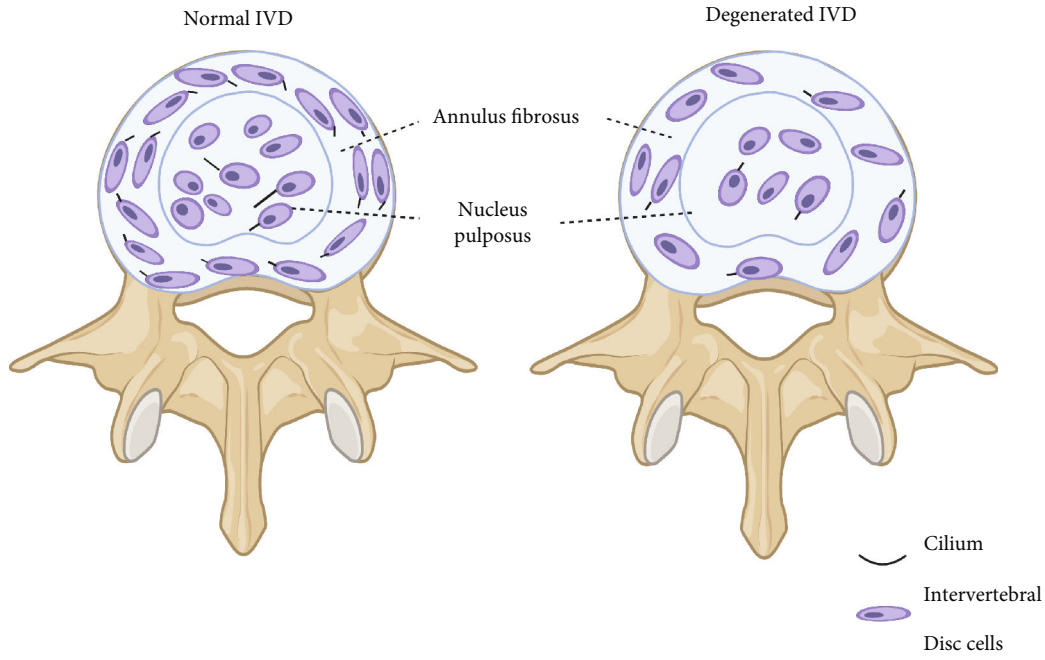

Figure 3: Cilium orientation of the normal and degenerated intervertebral disc (IVD). The IVD consisted of nucleus pulposus (NP), annulus fibrosus (AF), and endplate cartilage (EP). The primary cilia in AF are well organized and orientated: primary cilia were always projected from the inner sides of AF cells (near the NP), and they are oriented parallel to the long axis of the cells. The cilia in NP were disorganized and with varied cilium length. However, in the degenerated IVD, the cilia are disorganized in AF and cilium length and cilium incidence are reduced in both NP and AF.

chondrocytes when compared to healthy controls. All these suggested that primary cilia are closely related to OA, but how cilia changed and functioned during this process is still unclear.

To know how cilia functioned during articular cartilage and OA development, different cilium-related genes were deleted by genetic editing technology in the mouse model (Table 2). Deletion of IFT88 in cartilage causes several OA phenotypes with increased expression levels of degeneration markers, including MMP13, collagen type X, Adamts5, and Runx2 [8]. Similarly, the Bardet-Biedl syndrome 1 (Bbs1), Bbs2, or Bbs6 gene mutation mouse model developed OA-like cartilage abnormalities including proteoglycan loss, small surface fibrillation, marked atrophy of the cartilage, and increased MMP13 expression [42, 43]. Moreover, IFT88 deletion following surgical destabilization of the medial meniscus was found to have increased OARSI scores of cartilage damage mouse [43]. All these studies suggested that primary cilia are essential for cartilage development and prevent its degeneration.

Interleukin-1 (IL-1) is one of the most important inflammation media during OA formation and process. Wann and Knight [44] found that IL-1 can elongate the chondrocyte cilia via a PKA-dependent mechanism. Moreover, cilium loss can significantly attenuate IL-1-induced inflammatory response and alleviate the progression of OA. Interestingly, cilium elongation in response to IL-1 requires the accumulation of hypoxia-inducible factor-2α (HIF-2α) in cilia. Consistently, Yang et al. [45] reported that upregulated HIF-2α contributes to OA development through mediating the primary cilium loss. Mechanical stimulation was reported to be anti-inflammatory in many tissues. Recently, Fu et al. [46] reported that mechanical loading can suppress chondrocyte inflammatory induced by IL-1β via HDAC6-dependent modulation of tubulin leading to cilium disassembly during OA. Most recently, Fu et al. [47] revealed that osmotic-sensitive ion channel transient receptor potential vanilloid 4 (TRPV4), the key protein for mechanotransduction, localizes to the cilium plasma membrane. Mechanical, osmotic, or pharmaceutical activation of TRPV4 functioned as an anti-inflammatory agent during OA via regulating HDAC6-dependent modulation of ciliary tubulin. These results provided evidence that primary cilium is involved in an inflammatory process and it could be an important target for the treatment of inflammatory diseases such as OA.

Galectin 3 (Gal3) was found to be localized at the cilium base, and its absence causing cilium abnormalities is associated with disrupted epithelial cell polarity. Recently, Hafsia et al. reported that deletion of Gal3 in mouse can develop early onset of OA and exacerbate joint instability-induced OA via mitochondrial apoptosis [48].

2.4. Primary Cilia and IVDD. The IVDD occurs in more than 90% of the population older than 50 years [49]. The currently available treatments only provide symptomatic relief from pain [50–53], and these measures cannot decelerate or prevent the progression of degeneration of the intervertebral disc (IVD). Understanding the exact etiology of IVDD and finding the solution to the etiology is the key to cure this disease. A variety of risk factors, such as abnormal mechanical loading, aging, and smoking have been regarded as important factors causing IVDD [54]. Among these factors, abnormal mechanical loading has been considered the major contributor. Although the exact mechanism that abnormal mechanical loads affect cell behaviors in IVD remains unknown, previous studies have revealed that primary cilia played critical roles during cell mechanosensation and mechanotransduction. In the last several years, some scholars have tried to investigate the cilia in IVD and find the existence of primary cilia in IVD. The IVD consisted of nucleus pulposus (NP), annulus fibrosus (AF), and endplate cartilage (EP). Donnelly et al. [55] first attempted to detect cilia in rat IVDs by using multiphoton microscopy and found positive staining in the AF. Zheng et al. [56] examined the primary cilia in the mouse and human NP cells in vitro after 48 h of serum starvation. They furtherly found that parathyroid hormone 1 receptor (PTH1R) is expressed in primary cilia of mouse and human NP cells and knockout PTH1R or cilia in the NP cells result in significant IVDD and blunt the effect of parathyroid hormone on attenuation of aged discs.

Recently, we carefully reported that primary cilia are present in the mouse IVD with the cilia-GFP and ARL13B-mCherry;Centrin2-GFP cilium dual reporter-expressing transgenic mouse model [57, 58]. With these two mouse models, we found that the primary cilium length was 0.5-15 μm in the NP and 0.5-3.5 μm in the AF. There are 33.62% of NP cells and 36.1% of AF cells that were ciliated in the mouse's third and fourth lumbar IVD (Figure 3). The NP is derived from the embryonic node and notochord during the development process. Leftward-directed fluid flow, which is produced in embryonic node cilium movement, was regarded as essential for left-right axis determination in mice. Consistently, in our previous study [58], about 2% of cilia with the irregular movement were identified in mouse NP. However, the cilium movement type is different from the clockwise movement of cilia in embryonic notochord/node cells [59]. Interestingly, with the ARL13B-mCherry;Centrin2-GFP mouse model, we find that the cilia in AF are well organized and orientated: primary cilia were always projected from the inner sides of AF cells (near the NP), and they are oriented parallel to the long axis of the cells [58]. To further study the function of primary cilia in IVD, we crossed IFT80$^{fl/fl}$ mice with Col2a1-creERT mice and Col1a2-creERT to impair the primary cilia in IVD. As a result, we find that the deletion of IFT80 can cause an early onset of the IVDD phenotype, characterized by disorganized and decreased growth plate, EP, internal AF (IAF), less compact and markedly decreased gel-like matrix in the NP, and disorganized outer AF (OAF) with thinner, loosened, and disconnected fiber alignment. All these demonstrated that the primary cilia are essential for the maintenance of IVD development [57].

It was reported that NP in IVD can adapt to their physiologically hyperosmotic microenvironment and mediated osmoregulation through the nuclear factor of activated T cell 5 (NFAT5), a tonicity-responsive enhancer-binding protein. As an osmosensor in the natural world, whether cilia contribute to NP cell osmoadaptive response in IVD remains unknown. Choi et al. [60] found that primary cilia in NP cells could change their length in response to osmotic stimulation. However, when silencing of IFT88 or Kif3a to impair primary ciliogenesis did not affect hyperosmotic upregulation of TonEBP, then they concluded that primary cilia in NP have not participated for TonEBP-dependent osmoadaptive response.

In addition, we found that primary cilia in NP reduced during aging and injured induced IVDD and significantly increased during repair, indicating that primary cilia are essential for IVD repair or regeneration [57]. Thus, promoting ciliogenesis in AF and NP progenitors could be a promising target in the treatment of IVDD.

2.5. Primary Cilia and Idiopathic Scoliosis (IS). Scoliosis manifests as spine abnormal three-dimensional curvature, and around 10% of all scoliosis are idiopathic [61]. IS are always born with a normal spine, and the abnormal curvature may begin evident in the adolescent during growth,

Table 3: Primary cilium-related gene and scoliosis.

Gene	Scoliosis phenotype	Function in cilium biology
TBX6	Congenital and idiopathic scoliosis in humans [68, 69]	Affects morphology and motility of nodal cilia in mice and zebrafish [102, 103]
LBX1	Idiopathic scoliosis association in several ethnic groups, confirmed using different approaches [104–107]	Deleted in a mouse model of the primary ciliary dyskinesia gene [108]
GPR126	Scoliosis in humans and mice [109–111]	Essential for the development of myelinated axons [70, 112]
PAX1	Congenital and idiopathic scoliosis in humans and mice [71–73]	Other family members are associated with cilium signaling pathways [113–115]
POC5	Idiopathic scoliosis in humans [74]	Essential for centriole structure [116, 117]
KIF6	Idiopathic-type curvature in zebrafish [75]	Predicted to be involved in ciliary function or structure [118]
PTK7	Idiopathic-type curvature in zebrafish [76]	Role in cilium orientation in zebrafish [77]
FGF3	Idiopathic scoliosis in a KO mouse model; scoliosis in a human case report carrying loss-of-function mutation in the gene [78, 79]	Affecting the organization of chondrocyte primary cilia in the growth plate in mice [80]
SHP2	Idiopathic scoliosis in a KO mouse model [81, 82]	The length of primary cilia reduced in mutated mice [81]
IFT88	Idiopathic-type curvature in human and zebrafish [64, 83]	Essential for ciliogenesis [83]
IFT20	Idiopathic-type curvature in zebrafish [83]	Essential for ciliogenesis [83]
Arl13b	Idiopathic-type curvature in zebrafish [83]	Essential for ciliogenesis [83]
Yap	Idiopathic-type curvature in zebrafish [83]	Interacts with cilia [83]

and IS is diagnosed by excluding congenital defects and other causes of abnormal spine curvatures, such as intervertebral disc or vertebral development defects or other syndromes.

The exact etiology of IS is largely unknown due to its phenotypic and genetic heterogeneity. It is believed that heredity, melatonin, and biomechanical factors of the musculoskeletal system play an important role in its occurrence and progress. Among these factors, the research on genetic correlation has been done by many scholars. Although many genome-wide association studies (GWAS) have found some potential locus mutations, no clear and definite biological mechanism for IS has emerged so far. Nowadays, more and more scholars believed that IS is a complex consequence of genetic variations coupled with biomechanical factors that are affected by individual behavioral patterns. As an organ that bears the main force of the body, the contribution of biomechanics to IS is also valued by researchers. Mechanical loading can alter primary cilium incidence, length, and orientation of chondrocytes, and cilium direction is proven to affect the growth direction in growth plates [57]. The disorganized growth plates were also reported as one of the basic pathology changes in IS [62, 63]. Moreover, several human ciliopathies manifested as skeletal disorders, such as asphyxiating thoracic dystrophy syndrome [64]. Interestingly, the fact that asphyxiating thoracic dystrophy syndrome patients combined with scoliosis makes people believe that IS is a ciliopathy and that the genetic architecture of IS may involve cilium function [64].

Grimes et al. [10] revealed that protein tyrosine kinase-7 (ptk7) mutant zebrafish, a faithful genetic model of IS, exhibits ependymal cell cilium development and cerebrospinal fluid flow defects. Transgenic reintroduction of Ptk7 in motile ciliated lineages prevents scoliosis in ptk7 mutants. Oliazadeh et al. [65] found that primary cilia are significantly elongated in bone cells of IS patients. These IS bone cells can differentially express osteogenic factors and mechanosensitive and signaling genes in response to mechanical stimulation, compared with control. Moreover, many scoliosis association genes [66, 67], for example, TBX6 [68, 69], LBX1, GPR126 [70], PAX1 [71–73], POC5 [74], KIF6 [75], PTK7 [76, 77], FGF3 [78–80], SHP2 [81, 82], IFT88 [64, 83], IFT20 [83], Arl13b [83], and Yap [83], are found to be associated with cilium function so far (Table 3). Therefore, primary cilia are though important for IS development. The monocilia, presenting on the ventral surface of the mouse node, play an important role in determining human left-right symmetry. In addition, the high prevalence of right thoracic IS indicated the possible relation between IS and primary cilia. Burwell et al. [67] think that should the leftward nodal flow of morphogens—which affect precursors of the heart, great vessels, and viscera to create "handed asymmetry"—be extended by anomalous genetic/environmental factors to left-sided mesodermal precursors of vertebrae and ribs, an asymmetric skeletal anomaly may be imprinted. Such an anomaly may lead to relative left costovertebral physeal overgrowth that triggers right thoracic IS and anomalous upper limb length asymmetry. Coincidentally, Burwell et al. found that 50% of patients with dextrocardia had curves convex to the right as it showed in primary ciliary dyskinesia [67]. Schlösser et al. [84] found that the prevalence of scoliosis (Cobb > 10 degrees) and significant spinal asymmetry (Cobb 5–10 degree) were 8 and 23%, respectively, in 198 primary ciliary dyskinesia patients. It was further found that the convexity of the thoracic curve is predominantly to the right in normal organ anatomy and

to the left in patients with situs inversus totalis after the analysis of the scoliosis of 16 primary ciliary dyskinesia patients. We observed that around 10% of mice developed scoliosis in our cilium gene-knockout mouse model, even all the knockout mice combined with an extremely narrowed cage (unpublished). Although it was also reported that the rib cage abnormal development can result in progressive thoracic scoliosis [85], only around 10% of mice with the same genotyping developed scoliosis suggesting that the environmental factors or other factors may contribute to scoliosis. It will be interesting to investigate the role of cilium biology and environmental factors in the progress of idiopathic scoliosis in the future.

2.6. Primary Cilia and Tendon Disease. Tendons play vital roles in transferring our force from muscle to bone. Tendinopathy is a type of tendon injury and chronic tendon disease, and it is highly prevalent but has few treatment methods so far. Tendon bears dynamic tensile mechanical loading in normal conditions. As a mechanical sensitive organelle, the primary cilia have been found to exist in tendon. Primary cilia were observed in 64% of tenocytes in 3-week-old Sprague-Dawley rats, and they were aligned parallel to the collagen fibers and the long axis of the tendon [86].

Fang et al. [87] found that cilium incidence of tendon enthesis cells increased significantly between postnatal from 4.6% in one week old to 29.7% in two weeks old, and it decreased to 12.1% at 13 weeks old. However, they found a low level of ciliogenesis during the mouse postnatal stage in tendon midsubstance cells. To further know the role of primary cilia in tendon development, the $IFT88^{fl/fl}$ mouse was crossed with Scx-cre mouse for deletion of IFT88 in Scx-expressing cells. As a result, the growth of IFT88-knockout mice was slower, and it showed significantly lower body weights compared to controls. The tendon entheses had decreased structural properties (maximum force and stiffness) and increased material properties (stress and modulus) with drastically smaller cross-sectional areas in tendon entheses in 13-week-old IFT88-knockout mouse, which is an important feature for tendinopathy [88]. Considering that physical loading is an important driver of tendon formation or enthesis pathologies, primary cilia can be promising targets whose mechanosensitivity could potentially be tuned to prevent the progression of tendinopathy. However, how the cilia changed in the tendon during tendinopathy is still largely unknown. Further study on cilia of tendinopathy should be investigated in the future.

2.7. Primary Cilia and Other Skeletal Disorders. Ciliary gene mutation can impair skeletal development and cause a group of rare inherited chondrodysplasias diseases. All ciliary chondrodysplasias are characterized by developmental skeletal defects, mainly affecting limbs, ribs, spine, and craniofacial skeleton. They can be subdivided into different groups of severity, clinical phenotype, and underlying genetic defects.

2.7.1. Short-Rib-Polydactyly Syndromes. It is a group of perinatal lethal skeletal dysplasia characterized by severe narrowing of the thorax leading to pulmonary hypoplasia, short limbs, and polydactyly. It is caused by NEK1, DYNC2H1, and other gene mutations [89].

2.7.2. Oral-Facial-Digital Syndrome. Oral-facial-digital syndrome is characterized by pre- and postaxial polydactyly of the hands and feet, tibia hypoplasia, and oral and facial defects. Mutations in TCTN3 may cause up to 50% of all cases [90].

2.7.3. Asphyxiating Thoracic Dystrophy. Asphyxiating thoracic dystrophy (Jeune's syndrome) is characterized by a variable degree of rib shortening, typical pelvis configuration with trident acetabular roof, and acetabular spurs and rarely exhibits polydactyly. Asphyxiating thoracic dystrophy usually is caused by mutations in DYNC2H1, IFT40 and IFT80 [7, 91].

2.7.4. Mainzer-Saldino Syndrome. Mainzer-Saldino syndrome is characterized by cone-shaped epiphyses of the hand, retinal disease, and deterioration of renal function. A narrow ribcage, craniosynostosis, and liver involvement can present in some cases. Causative mutations in IFT140 have been identified in this disease [92].

2.7.5. Cranial-Ectodermal Dysplasia. Cranial-ectodermal dysplasia is a combination of dolichocephaly due to craniosynostosis of the sutura sagittalis, epicanthus, very thin, sparse, and slow-growing hair, tooth abnormalities, brachydactyly, and short rib. Cranial-ectodermal dysplasia is genetically heterogeneous with causative mutations found in IFT122, IFT43, WDR19, and WDR35 [93].

2.7.6. Ellis-van Creveld Syndrome. Ellis-van Creveld syndrome (EVC) is characterized by acromelic dwarfism, polydactyly of the hands' dysplastic nails, tooth abnormalities, and cardiac defects. Biallelic causative mutations in EVC1 and EVC2 have been identified with mutations in EVC1 accounting for 75% and mutations in EVC2 accounting for 25% of the cases [94].

2.7.7. Weyers Acrofacial Dysostosis. Weyers acrofacial dysostosis (Curry-Hall syndrome) is characterized by a milder phenotype of polydactyly, dentition anomalies, and dystrophic nails. Dominant mutations in EVC1 and EVC2 have been found to cause Weyers acrofacial dysostosis [95].

3. Conclusion

Numerous studies have shown a variety of functional and structural relationships between primary cilia and physiological as well as pathological aspects of the skeletal system. In this review, we provide insight into the role of primary cilia in skeletal disease and show evidence that the primary cilia may be a promising target of clinical intervention for bone/cartilage tumor, OA, IVDD, scoliosis, osteoporosis, and cilium-related skeletal disease.

Data Availability

All the data are included within the article.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

XL and ZL searched literatures, collected data, and prepared the initial manuscript. All coauthors took part in the revision of the review. Xinhua Li and Song Guo contributed equally to this work.

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