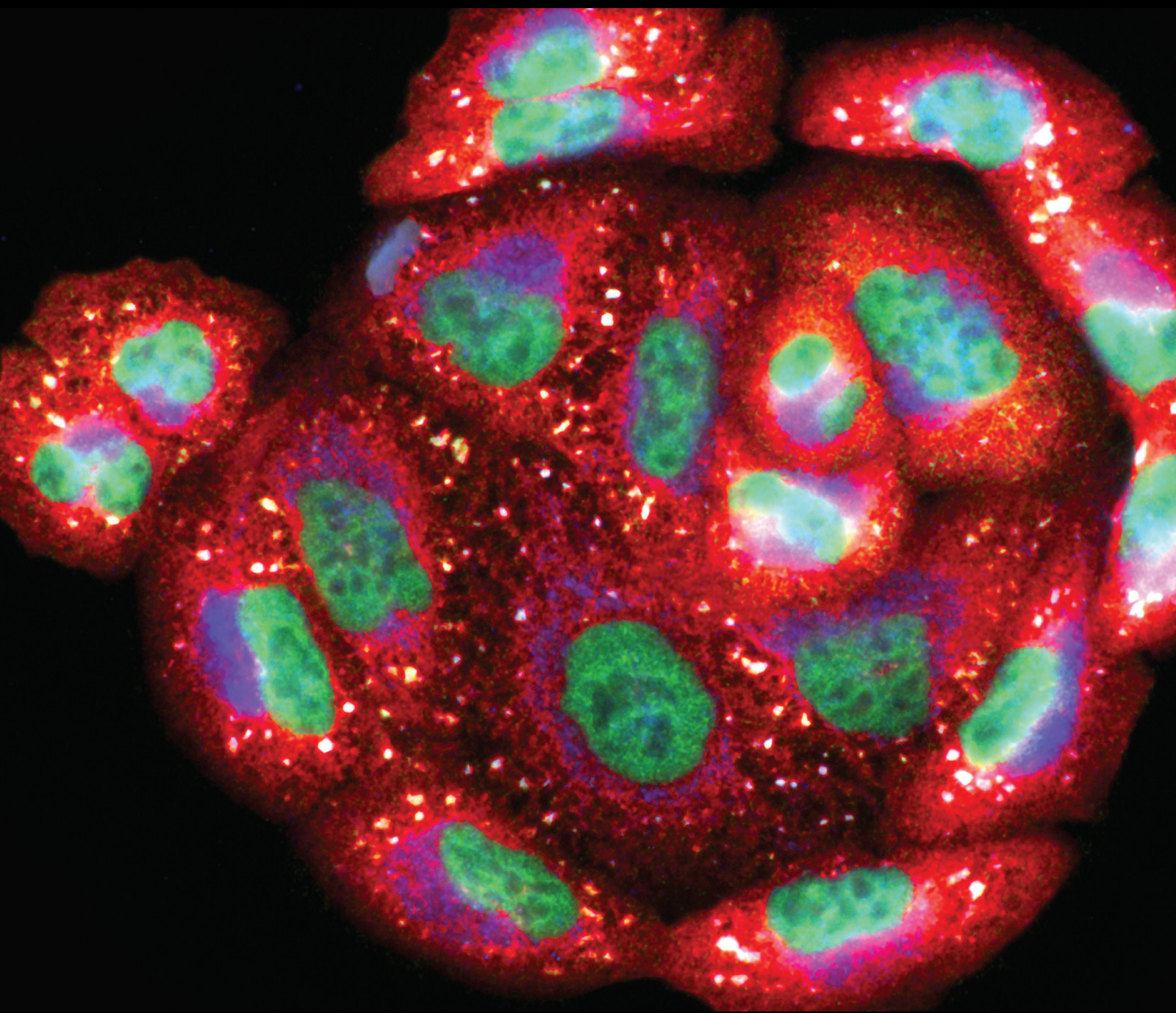
Oxidative Medicine and Cellular Longevity

Oxidative Stress and Exosomes in Aging Disease

Lead Guest Editor: Yuan Xiong
Guest Editors: Yong Xu and Adriana C. Panayi



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Retraction

Retracted: MicroRNA-133b Inhibition Restores EGFR Expression and Accelerates Diabetes-Impaired Wound Healing

Oxidative Medicine and Cellular Longevity

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(1) Discrepancies in scope
(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
(4) Inappropriate citations
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Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] H. Zhong, J. Qian, Z. Xiao et al., "MicroRNA-133b Inhibition Restores EGFR Expression and Accelerates Diabetes-Impaired Wound Healing," *Oxidative Medicine and Cellular Longevity*, vol. 2021, Article ID 9306760, 14 pages, 2021.

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Volume 2024, Article ID 9869257, 1 page
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Retraction

Retracted: Mesenchymal Stem Cell-Derived Exosomes and Their Potential Agents in Hematological Diseases

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2024, Article ID 9860941, 1 page
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Retraction

Retracted: Periodontitis Exacerbates Benign Prostatic Hyperplasia through Regulation of Oxidative Stress and Inflammation

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Oxidative Medicine and Cellular Longevity
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https://doi.org/10.1155/2024/9808072

Retraction

Retracted: Platelet-Rich Plasma-Derived Exosomal USP15 Promotes Cutaneous Wound Healing via Deubiquitinating EIF4A1

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Retraction

Retracted: Exosome: Function and Application in Inflammatory Bone Diseases

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Retraction

Retracted: MicroRNA-133b Inhibition Restores EGFR Expression and Accelerates Diabetes-Impaired Wound Healing

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Research Article

MicroRNA-133b Inhibition Restores EGFR Expression and Accelerates Diabetes-Impaired Wound Healing

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Diabetic foot ulcers (DFUs) are caused by impairments in peripheral blood vessel angiogenesis and represent a great clinical challenge. Although various innovative techniques and drugs have been developed for treating DFUs, therapeutic outcomes remain unsatisfactory. Using the GEO database, we obtained transcriptomic microarray data for DFUs and control wounds and detected a significant downregulation of epidermal growth factor receptor (*EGFR*) in DFUs. We cultured human umbilical vein endothelial cells (HUVECs) and noted downregulated *EGFR* expression following high-glucose exposure *in vitro*. Further, we observed decreased HUVEC proliferation and migration and increased apoptosis after shRNA-mediated *EGFR* silencing in these cells. In mice, *EGFR* inhibition via focal EGFR-shRNA injection delayed wound healing. Target prediction analysis followed by dual-luciferase reporter assays indicated that microRNA-133b (miR-133b) is a putative upstream regulator of *EGFR* expression. Increased miR-133b expression was observed in both glucose-treated HUVECs and wounds from diabetes patients, but no such change was observed in controls. miR-133b suppression enhanced the proliferation and angiogenic potential of cultured HUVECs and also accelerated wound healing. Although angiogenesis is not the sole mechanism affected in DFU, these findings suggest that the miR-133b-induced downregulation of *EGFR* may contribute to delayed wound healing in diabetes. Hence, miR-133b inhibition may be a useful strategy for treating diabetic wounds.

1. Introduction

Diabetic wounds cause serious economic, physical, and psychosocial burdens among diabetes patients and often show poor healing due to underlying vascular lesions [1, 2]. Diabetic foot ulcers (DFUs) are a major health problem, affecting 9.1–26.1 million individuals with diabetes globally every year. DFUs are characterized by impairments in angiogenesis—a key step in wound healing [3–5]. Given how critical the vascular endothelium is for wound healing, the identification of mechanisms preventing a normal angiogenic response in DFUs is crucial for developing effective treatments.

The epidermal growth factor (EGF) receptor (EGFR; ErbB-1; HER1 in humans) is primarily expressed in epithelial cells and promotes cellular proliferation, migration, and survival [6, 7]. Moreover, the interaction between EGFR and its main ligand EGF results in EGFR signaling, which can also promote angiogenesis [7, 8]. There is robust evidence that angiogenesis is also modulated by several miRNAs that influence gene expression in endothelial cells [9–13]. For instance, a study showed that exosomal miR-21-5p acts on endothelial cells to promote vascular repair by suppressing the expression of thrombospondin-1, an angiogenesis inhibitor [14].

In the present study, we analyzed available microarray data and detected a significant downregulation of *EGFR* expression in DFUs. Based on this finding, we explored miRNA databases to identify putative upstream regulators of *EGFR* expression. Further, we conducted experiments in human vascular endothelial cells (HUVECs) and diabetic wounds in mice to assess how miR-133b affects endothelial cell function and wound healing.

2. Materials and Methods

2.1. Microarray and Bioinformatics. Microarray data for six and three individuals with and without diabetes, respectively, were obtained from a microRNA database in the Gene Expression Omnibus repository (GEO, http://www.ncbi.nlm.nih.gov/geo;GSE80178). The identification of differentially expressed genes (DEGs) was performed using the inbuilt GEO2R function at default settings based on a threshold of $p < 0.05$. Changes in mRNA expression were visualized using heatmaps created with the R package pheatmap. Subsequently, a protein-protein interaction (PPI) network was created after importing DEGs into the STRING database. Cytoscape 3.7.2 was used to calculate the degree, betweenness, and closeness of PPI genes with the help of the CentiScaPe 2.2. plugin. Finally, GO and KEGG pathway enrichment analyses were performed using DAVID [15], and the top three terms within each GO category and KEGG pathways were identified. The top ten genes (highest degree values) were visualized with ggplot2 on R [16] and considered hub genes. GOplot was used to visualize enrichment analysis results [17]. Chromosomal locations and the degree of connectivity for the top 50 DEGs were illustrated using the circlize package in R [18].

2.2. Ethics Approval. Both human and animal studies were conducted after approval from the Committees of Clinical Ethics of Huizhou First Hospital. All human studies were performed after obtaining informed consent from each participant.

2.3. Blood and Skin Samples. Peripheral blood samples were collected from patients at the Huizhou First Hospital (12 healthy volunteers and 12 with DFUs) between September 2016 and October 2018. Skin samples were also collected from patients at this center during the same period (6 DFU patients and 6 emergency foot trauma patients without diabetes). mRNA expression was examined in all blood and skin samples.

2.4. Murine Wound Model. Male C57BL/6J mice (age, 6–7 weeks; weight, 25–30 g) and male db/db mice (BKS.Cg-Dock7m +/+ Leprdb/J strain) were procured. All db/db mice were examined and found to be mildly to severely diabetic.

Before the induction of the wound model, all animals were anesthetized using pentobarbital sodium (50 mg/kg i.p.; Sigma-Aldrich, MO, USA). After shaving, full-thickness excisional skin wounds (diameter, 10 mm) were induced on their upper back. The mice were subsequently randomized into four groups ($n = 6$ mice/group) and received subcutaneous injections of EGFR shRNA, negative control (NC) shRNA, or mi-133b agonist/antagonist mimics at four sites surrounding the wound (25 μL/site). The injections were administered on days 0, 3, 5, 7, 9, and 11 postwound induction. On days 0, 3, 5, 7, 10, and 14, the wounds were imaged and measured using a caliper rule.

2.5. Wound Closure. The wound closure rate was calculated on days 0, 3, 5, 7, 10, and 14 postwound induction using digital photographs, which were then analyzed using ImageJ software (NIH, USA).

2.6. Cell Culture and Transfection. HUVECs (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI 1640 medium (Thermo Fisher Sci., Inc., MA, USA) containing 10% FBS (Gibco, New York, USA). Transfection was performed using Lipofectamine 3000 (Thermo Fisher Sci., Inc.) based on the manufacturer's instructions in cultured cells (37°C; 5% CO_2 and 95% humidity). The agomiR-133b and antagomiR-133b constructs (100 nM) were obtained from GenePharma (Shanghai, China) and transfected into cells. EGFR-specific shRNA (shEGFR) was cloned into pSicoR vectors. For glucose stimulation assays, HUVECs were cultured in 20 mM D-glucose for 72 h.

2.7. CCK-8 Assay. HUVECs (5×10^3) were cultured for 24, 48, or 72 h in 96-well plates. Subsequently, CCK-8 reagent (#96992, Sigma-Aldrich, MO, USA) was added to each well containing serum-free medium for 2 h. Absorbance was measured at 450 nm.

2.8. Transwell Migration Assay. Transwell inserts (#140629, Thermo Fisher Sci.) with 8 μm pore filters were used to examine transwell migration. HUVECs in low serum (5% FBS) medium were added to the upper chamber (1×10^4 cells per insert). Complete medium (500 μL; containing 10% FBS) supplemented with different treatment agents was added to the lower chamber. After 12 h of incubation, cells adhering to the upper membrane surface were expunged using cotton swabs. Cells showing migration onto the lower surface were stained using 0.5% crystal violet, and counts were obtained from three random fields.

2.9. Luciferase Assay. Positions 50–56 in the 3′UTR of *EGFR* mRNA that contained the putative miR-133b target site were identified using TargetScan. Subsequently, using cDNA obtained from HUVECs, this region was PCR-amplified and ligated into a pGL3-basic vector (Promega, Madison, WI, USA). The pGL3-*EGFR* 3′UTR-mutant (Mut) construct was synthesized by inserting two site mutations within the potential target sequence of miR-133b using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, Inc., CA, USA). These two constructs were independently transfected into HUVECs (200 ng) along with the *Renilla* plasmid using Lipofectamine 3000 (Thermo Fisher Sci., Inc.). Subsequently, miR-NC mimic, agomiR-133b, or antagomiR-133b (48 h at 37°C; 10 nM) were transfected into cells using kits supplied by Shanghai GenePharma Co., Ltd. Relative luciferase activity was evaluated based on the firefly/*Renilla* activity ratio using a dual-luciferase reporter assay system (Promega).

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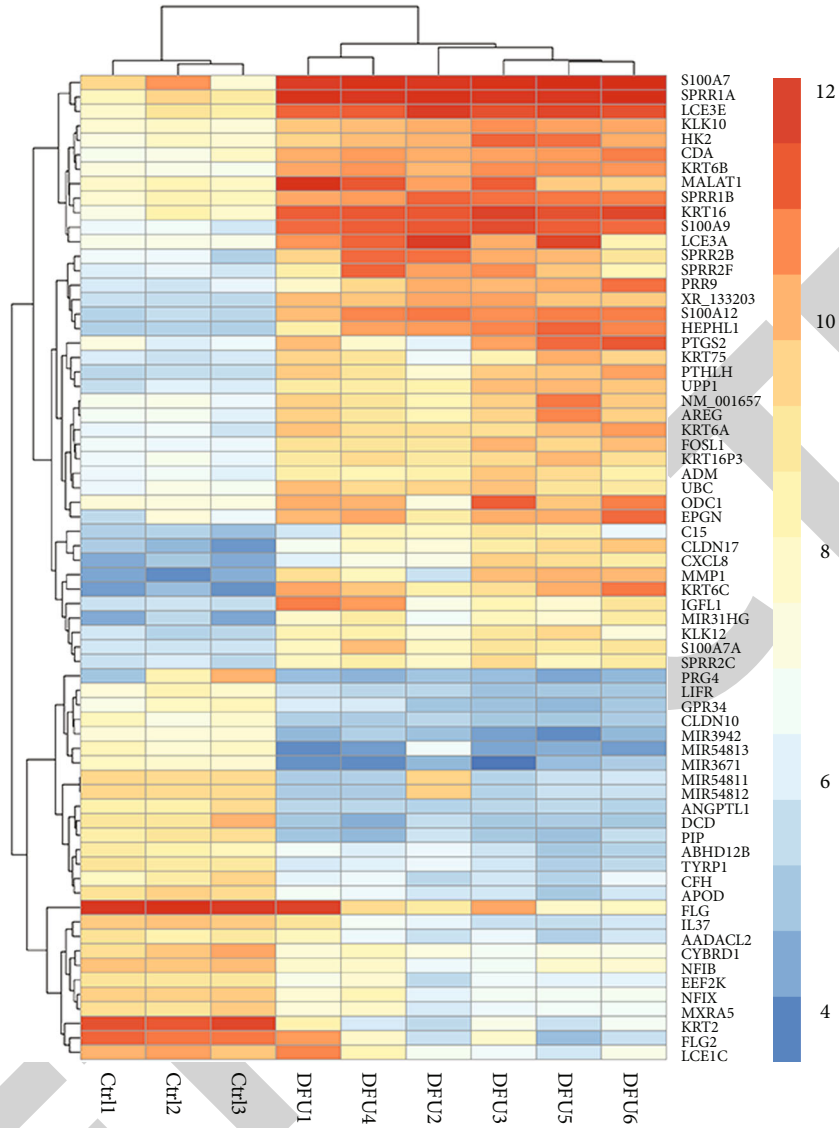

(a)

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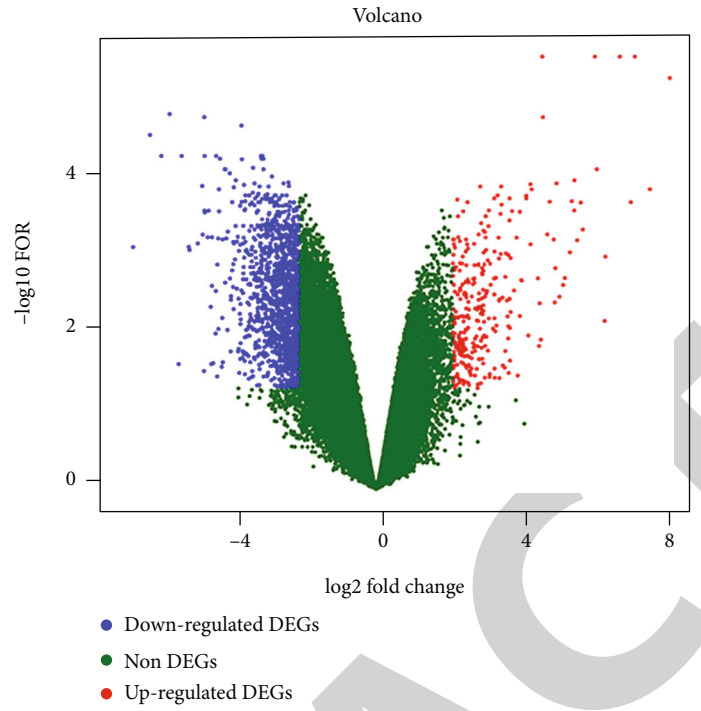

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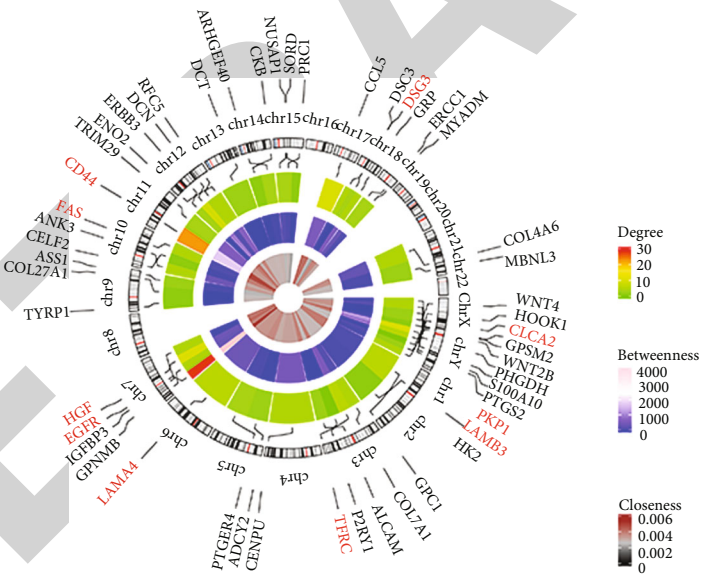

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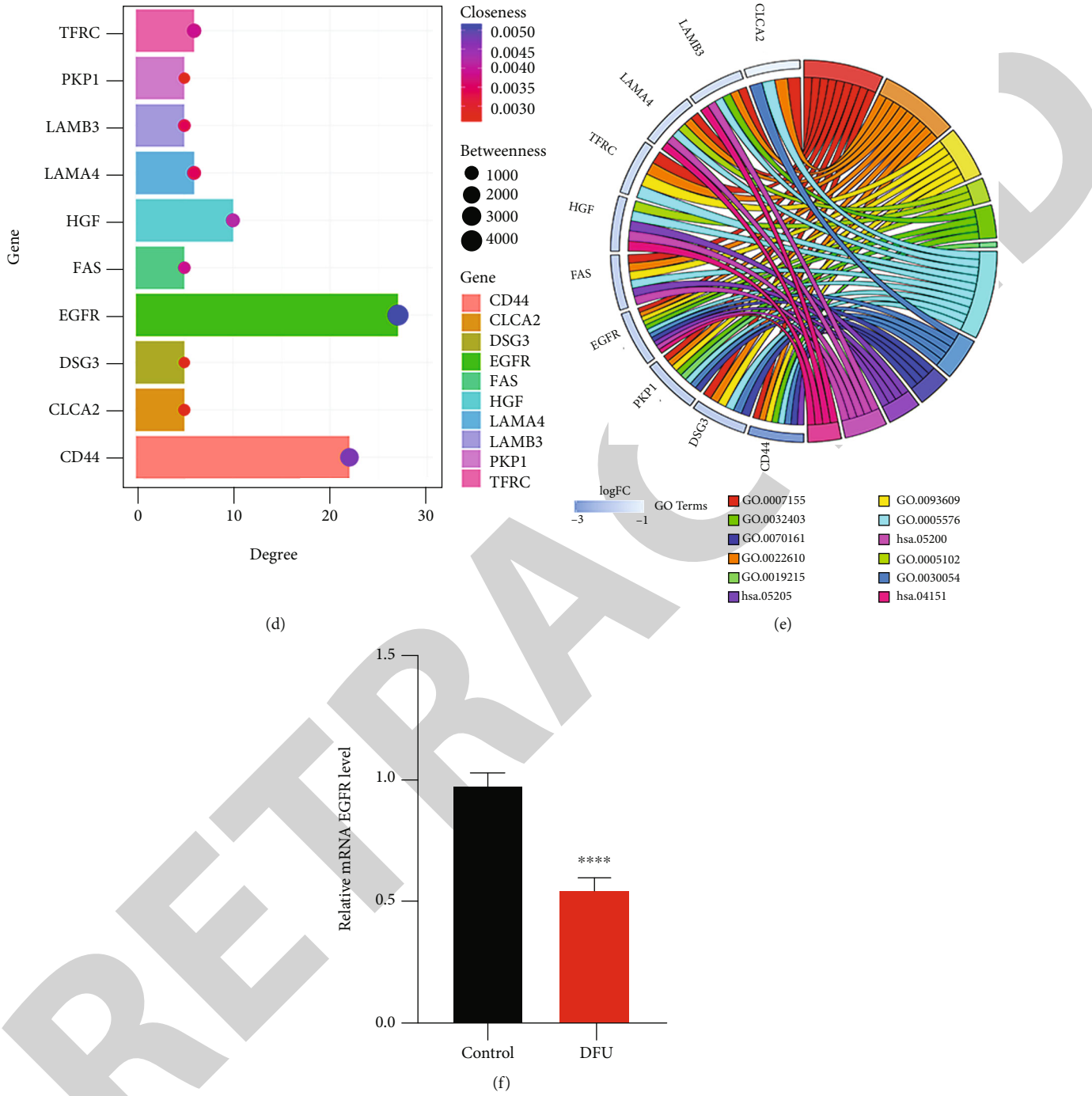

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Figure 1: *EGFR* expression is decreased in DFUs. (a) Hierarchical clustering heat map constructed using GSE80178 data. (b) Volcano plot based on GSE80178 data. (c) Degree centrality analysis of the top 50 degree-filtered genes from the DEG PPI network and corresponding chromosomal positions. The top 10 hub genes are highlighted in red. (d) Distribution of degree, betweenness, and closeness of the top 10 hub genes. (e) Enrichment analysis findings for the top 10 genes in the PPI network. (f) Expression of *EGFR* in skin tissues from non-DFU ($n = 6$) and DFU patients ($n = 6$) measured using qRT-PCR analysis.

2.10. qRT-PCR Analysis. First, total RNA was isolated from cells and tissues using the TRIzol reagent (Thermo Fisher Sci., Inc.) and then reverse transcribed into cDNA using the ReverTra Ace qPCR RT Master Mix (Toyobo Life Science, Tokyo, Japan) based on the manufacturer's instructions (15 min at 42°C, followed by 5 min at 98°C; reaction volume = 20 μL). The qPCR thermocycling conditions were as follows: initial denaturation, 95°C for 30 s; 40 cycles at 95°C for 5 s, and 60°C for 30 s (reaction volume = 25 μL). *GAPDH* was selected as the internal control. Relative miRNA expression was calculated using the $2^{-\triangle\triangle Ct}$ method. The primer sequences used were as follows:

miR-133b: F, GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACTAGCTG, R, CCGTTT

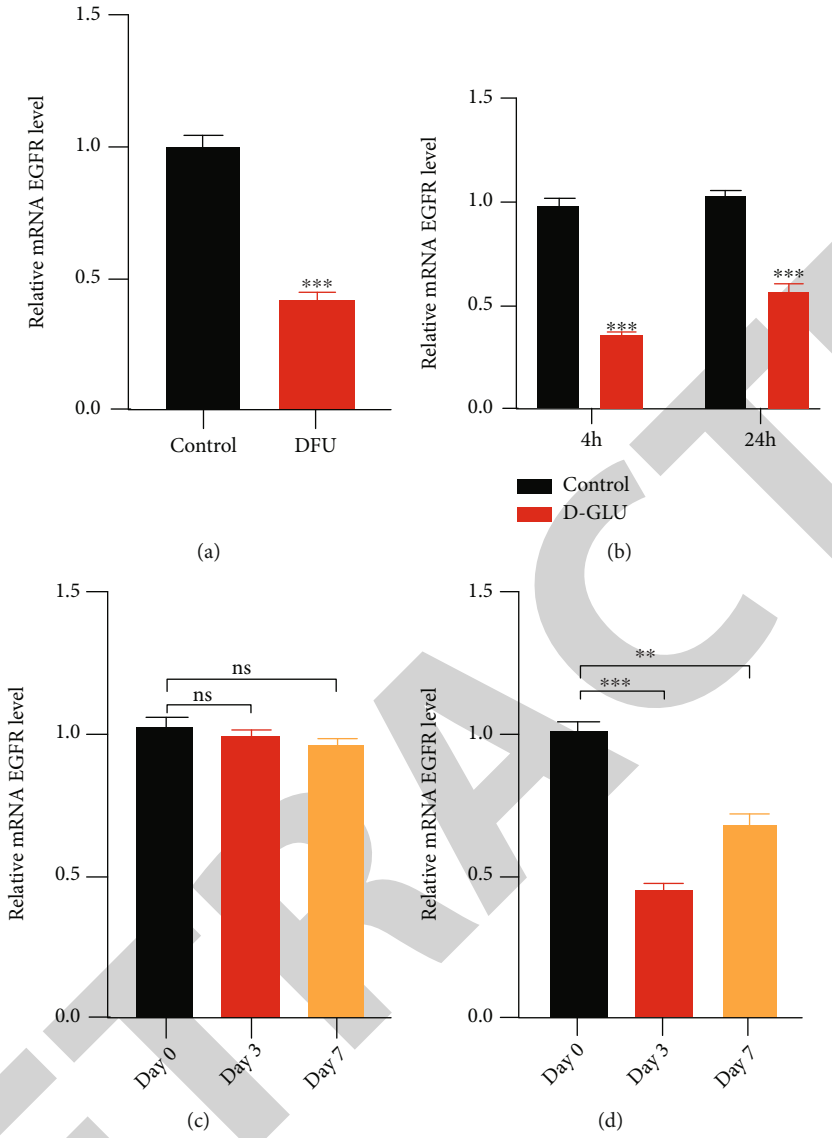

Figure 2: High-glucose exposure decreases EGFR expression in HUVECs. (a) Relative *EGFR* mRNA levels in peripheral blood from control volunteers and DFU patients ($n = 12$ per group). (b) Changes in relative *EGFR* mRNA levels in HUVECs, 4 and 24 h following exposure to 20 mM D-glucose. (c, d) Relative *EGFR* mRNA levels in wounds from nondiabetic (c) and diabetic mice (d) at 0, 3, and 7 d postwound induction.

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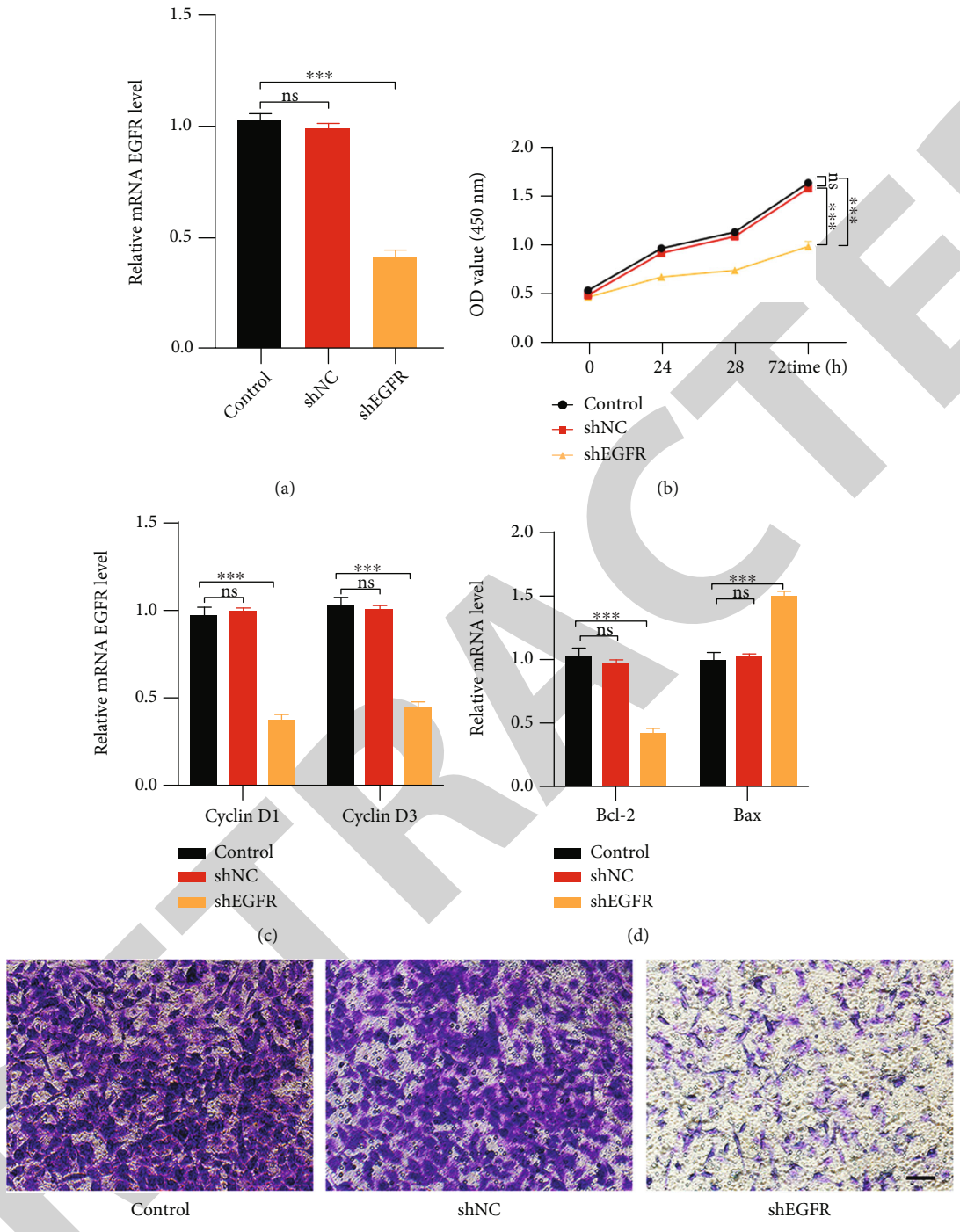
GGTCCCCTTCAAC; *Bcl-2*: F, GATAACGGAGGCTGGG ATGC, R, TCACTTGTGGCCCAGATAGG; *Bax*: F, CCCT TTTGCTTCAGGGTTTC, R, GAGACACTCGCTCAGC TTCTTG; *Cyclin D1*: F, TTGCCCTCTGTGCCACAGAT, R, TCAGGTTCAGGCCTTGCACT; *Cyclin D3*: F, CTGG CCATGAACTACCTGGA, R, CCAGCAAATCATGTGC AATC; *EGFR*: F, GGTCTT GAAGGCTGTCCAACG, R, CCTCAAGAGAGCTTGGTTGGG; *GAPDH*: F, CCGTTG AATTTGCCGTGA, R, TGATGACCCTTTTGGCTCCC.

2.11. Statistical Analyses. All experiments were conducted in triplicate. Data are presented as the mean ± SD. Student's *t* -tests or one-way ANOVAs with Tukey's post hoc test were applied for comparing two or more than two groups, respectively. GraphPad Prism 8.0 was used for all analyses. p values < 0.05 were considered significant ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

3. Results and Discussion

3.1. EGFR Expression Is Decreased in Diabetic Foot Ulcers. To search for DEGs in DFUs, the GSE80178 dataset, containing information on six DFUs and three nondiabetic foot skin samples, was retrieved from the GEO database. In total, there were 326 DEGs (88 upregulated and 238 downregulated in the DFU specimens) (Figures 1(a) and 1(b)). The degree centrality for the top 50 degree-filtered genes in the PPI network, as well as their chromosomal positions, is shown in Figure 1(c). The top 10 hub genes were *EGFR*, *CD44*, *HGF*, *TFRC*, *LAMA4*, *FAS*, *LAMB3*, *CLCA2*, *DSG3*,



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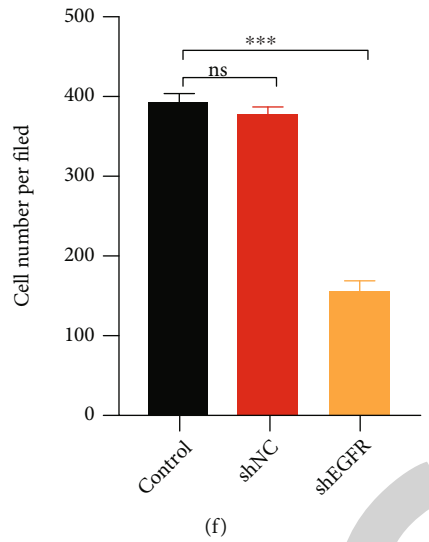
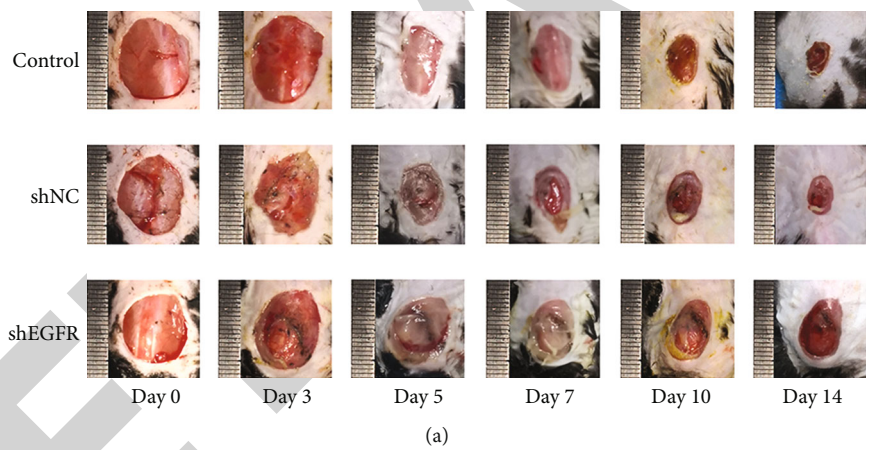
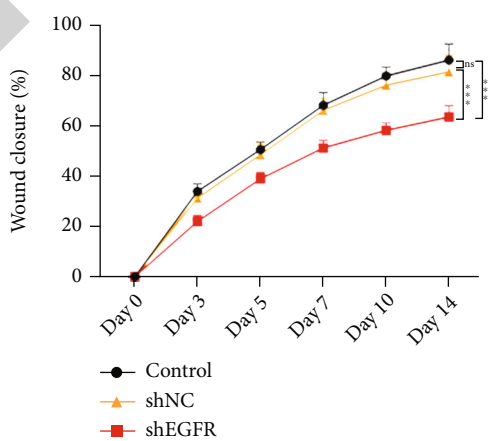


(f)

Figure 3: EGFR inhibition impairs HUVEC functions. (a) Relative *EGFR* mRNA expression in control, shNC, and shEGFR HUVECs. (b) Effect of EGFR shRNA treatment on HUVEC proliferation (CCK-8 assay). (c) qRT-PCR results showing *Cyclin D1* and *Cyclin D3* expression in HUVECs treated with EGFR shRNA. (d) qRT-PCR results showing *Bcl-2* and *Bax* expression in HUVECs treated with EGFR shRNA. (e, f) Transwell migration assay findings depicting the effect of EGFR shRNA on HUVEC migration. Scale bar: 100 μm.



(a)



(b)

Figure 4: EGFR inhibition delays wound healing *in vivo*. (a) Wound closure at various time points after different treatments. (b) Wound closure rates for the three experimental treatments.

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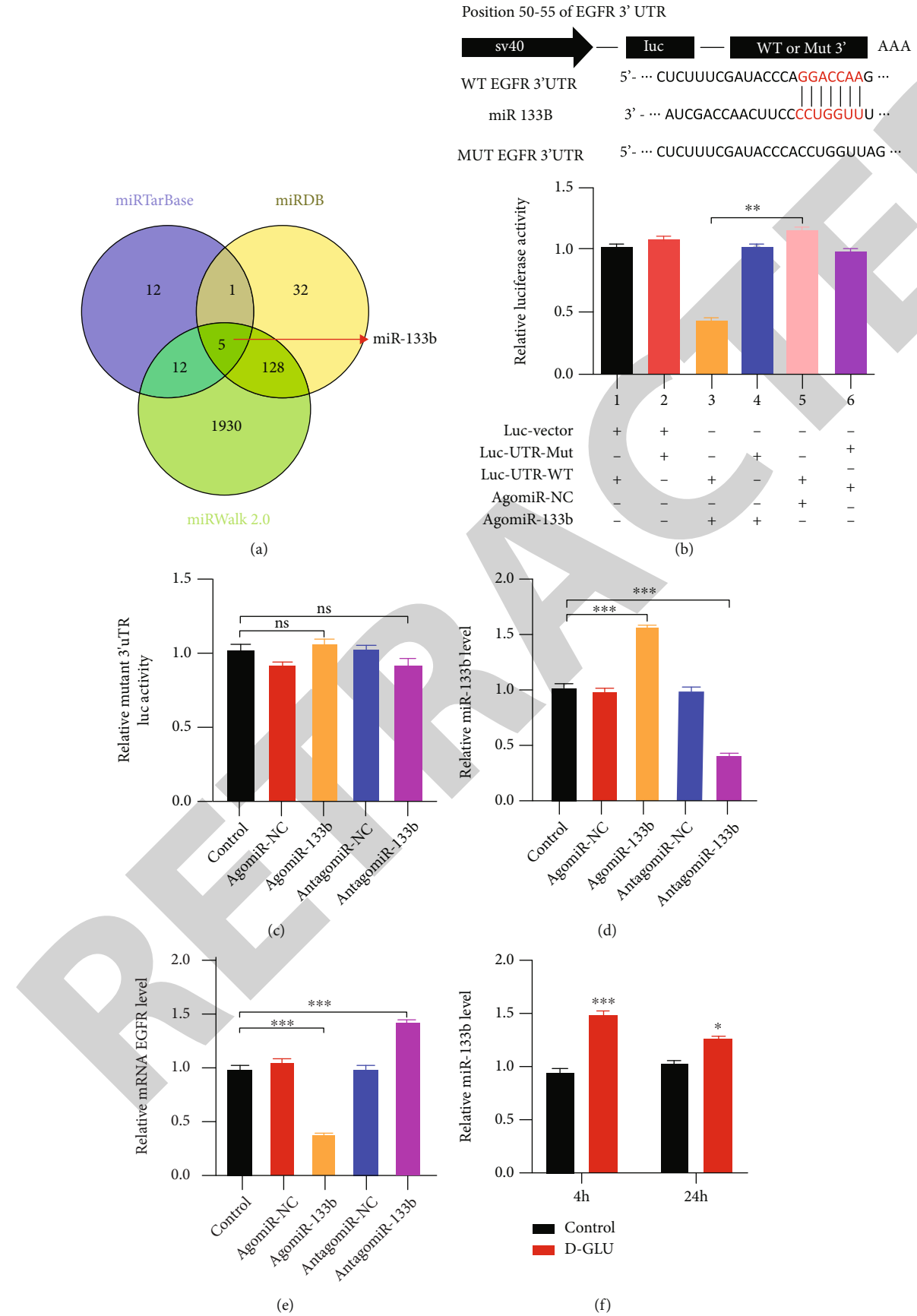

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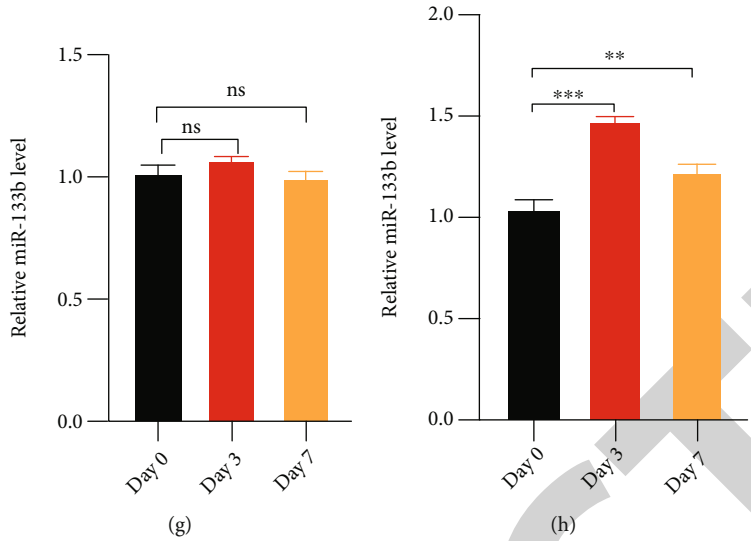

Figure 5: miR-133b is a potential regulator of *EGFR* expression. (a) Identification of miR-133b as a putative regulator of *EGFR* expression based on miRTarBase, TargetScan, and miRWalk target prediction analyses. (b, c) Luciferase reporter assay demonstrating that miR-133b binds to the 3′UTR of the *EGFR* mRNA. (d) Relative miR-133b levels in control and agomiR-NC-, agomiR-133b-, antagomiR-NC-, and antagomiR-133b-transfected HUVECs (qRT-PCR). (e) qRT-PCR for assessing the effect of miR-133b on *EGFR* expression. (f) Changes in relative miR-133b levels following exposure of HUVECs to 20 mM D-glucose for 4 and 24 h. (g, h) Relative miR-133b expression in wounds from nondiabetic (g) and diabetic mice (h) at 0, 3, and 7 d postwound induction.

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and *PKP1* (Figure 1(d)), of which the first three had the highest degree, betweenness, and closeness (*EGFR*, *CD44*, and *HGF*). The genes involved in the top three modules showing the highest MCODE scores were subjected to enrichment analysis. The DEGs were enriched under the following terms in the GO analysis: biological process: "cell adhesion," "biological adhesion," and "cell-cell adhesion"; molecular function: "receptor binding," "protein complex binding," and "intermediate filament binding"; and cellular component: "extracellular region," "cell junction," and "anchoring junction." In turn, the top three KEGG pathways enriched in these DEGs were "proteoglycans in cancer pathway," "pathway in cancer," and "PI3K-Akt signaling pathway." Results of enrichment analyses for the top hub genes are shown in Figure 1(e). The qRT-PCR results showed lower *EGFR* mRNA levels in DFU patients than in those without diabetes (Figure 1(f)).

3.2. High-Glucose Stimulation Reduces EGFR mRNA Expression in HUVECs. To validate the above findings, blood samples from 12 DFU patients and 12 healthy volunteers were analyzed using qRT-PCR. Results showed lower *EGFR* mRNA levels in DFU patients than in those without diabetes (Figure 2(a)). To understand if *EGFR* expression in HUVECs is altered in response to diabetic stimuli, qRT-PCR was performed on RNA extracted from cells exposed to hyperglycemic conditions (20 mM D-glucose). Results revealed that *EGFR* expression was decreased at 4 and 24 h after exposure to a high-glucose environment (Figure 2(b)). Moreover, *EGFR* expression was significantly lower in excisional wounds in diabetic mice than in nondiabetic controls at 3 and 7 d postwound induction (Figures 2(c) and 2(d)).

3.3. EGFR Inhibition Impairs HUVEC Functions. To understand how *EGFR* affects HUVEC activity *in vitro*, we transfected HUVECs with *EGFR*-specific shRNAs or a NC. We confirmed *EGFR* silencing using qRT-PCR analysis (Figure 3(a)). HUVEC proliferation was assessed using the CCK-8 assay, which indicated that *EGFR* knockdown decreased the proliferative capacity of cells (Figure 3(b)). Further, qRT-PCR assay showed that the expression of the proliferation-related genes *Cyclin D1* and *Cyclin D3* was decreased in EGFR-shRNA-treated cells (Figure 3(c)). Moreover, *EGFR* silencing also caused a change in the expression of the apoptosis-related genes *Bcl-2* and *Bax*, which were downregulated and upregulated, respectively, following transfection with EGFR-shRNA (Figure 3(d)). Finally, transwell migration assays also indicated that *EGFR* silencing weakened the migratory ability of HUVECs (Figures 3(e) and 3(f)).

3.4. EGFR Inhibition Delays Wound Healing In Vivo. In nondiabetic mice (Figure 4(a)), closure rate measurements revealed a significant delay in the healing of full-thickness back wounds after focal EGFR-shRNA injection (Figure 4(b)).

3.5. miR-133b Is a Potential Upstream Regulator of EGFR Expression. Using three different miRNA databases (miRTarBase, TargetScan, and miRWalk), we identified miR-133b as a putative *EGFR* partner and regulator (Figure 5(a)). Next, we verified the association between miR-133b and *EGFR* using a dual-luciferase assay in HUVECs transfected with 3′UTR-wild-type or 3′UTR-mutant sequences of *EGFR* mRNA. The results confirmed that miR-133b specifically binds to the predicted target site of *EGFR* mRNA (Figures 5(b) and

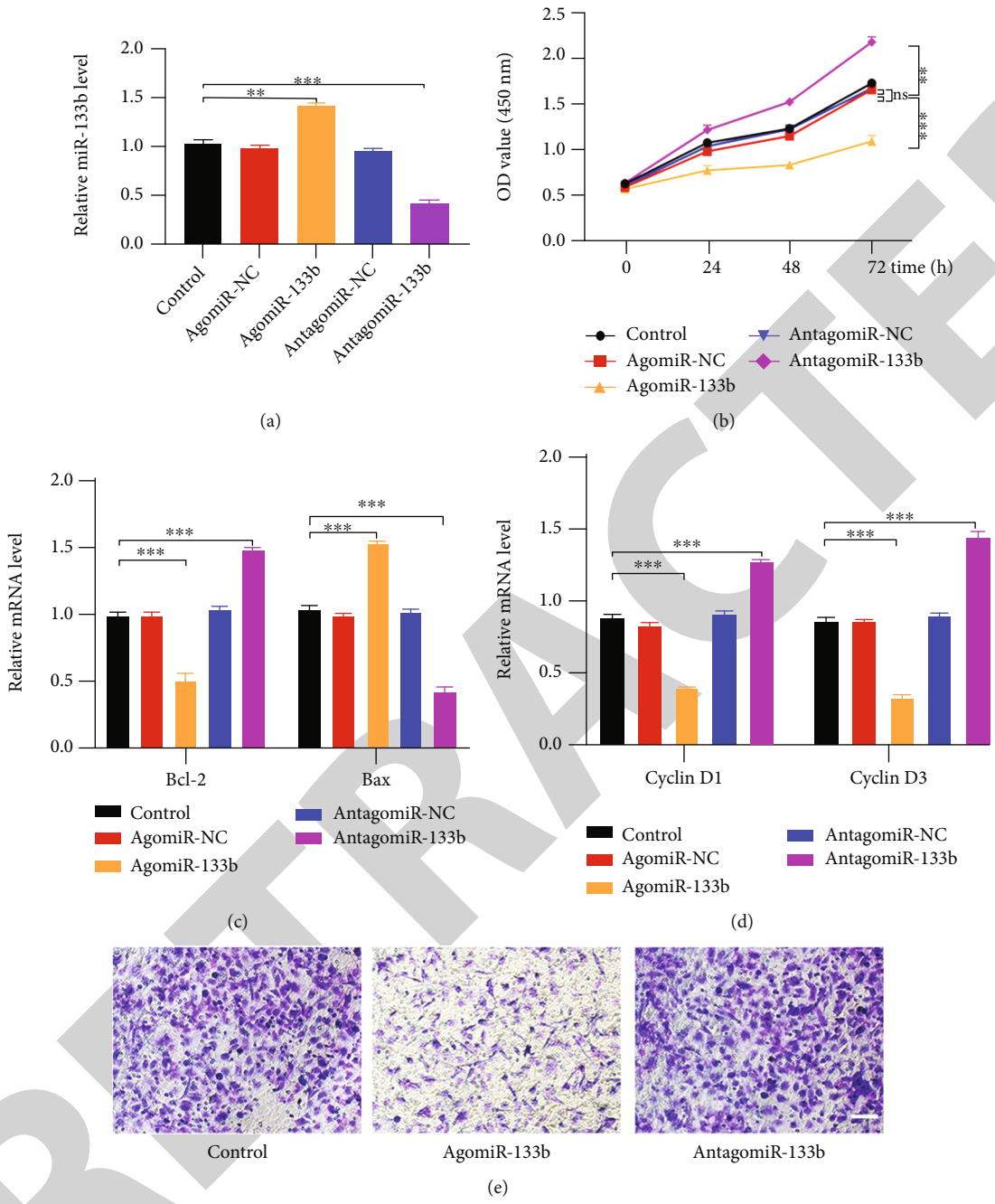

Figure 6: Suppression of miR-133b enhances HUVEC functions. (a) qRT-PCR for miR-133b expression in HUVECs transfected with miR-133b agonist and antagonist mimics. (b) CCK-8 proliferation assay showing HUVECs with enhanced and suppressed miR-133b expression. (c, d) Analysis of *Bcl-2* and *Bax* expression (c) and *Cyclin D1* and *Cyclin D3* expression (d) using qRT-PCR. (e) Transwell migration assay illustrating the effect of miR-133b inhibition on cell migration. Scale bar: 100 μm.

5(c)). Furthermore, agomiR-mediated miR-133b overexpression clearly suppressed the expression of *EGFR* (Figures 5(d) and 5(e)). To explore whether miR-133b expression is altered in response to a prodiabetic milieu, we examined *EGFR* levels in glucose-treated HUVECs using qRT-PCR. The results revealed increased miR-133b expression at 4 and 24 h after exposure to high-glucose conditions (Figure 5(f)). Similarly, miR-133b expression was also found to be significantly higher at 3 and 7 d postwound induction in diabetic mice than in nondiabetic animals (Figures 5(g) and 5(h)).

3.6. miR-133b Suppression Enhances HUVEC Functions. After verifying the modulatory effects of the specific miRNA agonist (agomiR-133b) and antagonist (antagomiR-133b) mimics on miR-133b expression (Figure 6(a)) using qRT-PCR, we evaluated how miR-133b affects HUVEC activity *in vitro*. CCK-8 assays showed that HUVEC proliferation was enhanced following transfection with antagomiR-133b (Figure 6(b)). In addition, qRT-PCR analyses for Cyclin D1, Cyclin D3, Bcl-2, and Bax revealed an increase in proliferation and reduction in apoptosis in HUVECs transfected

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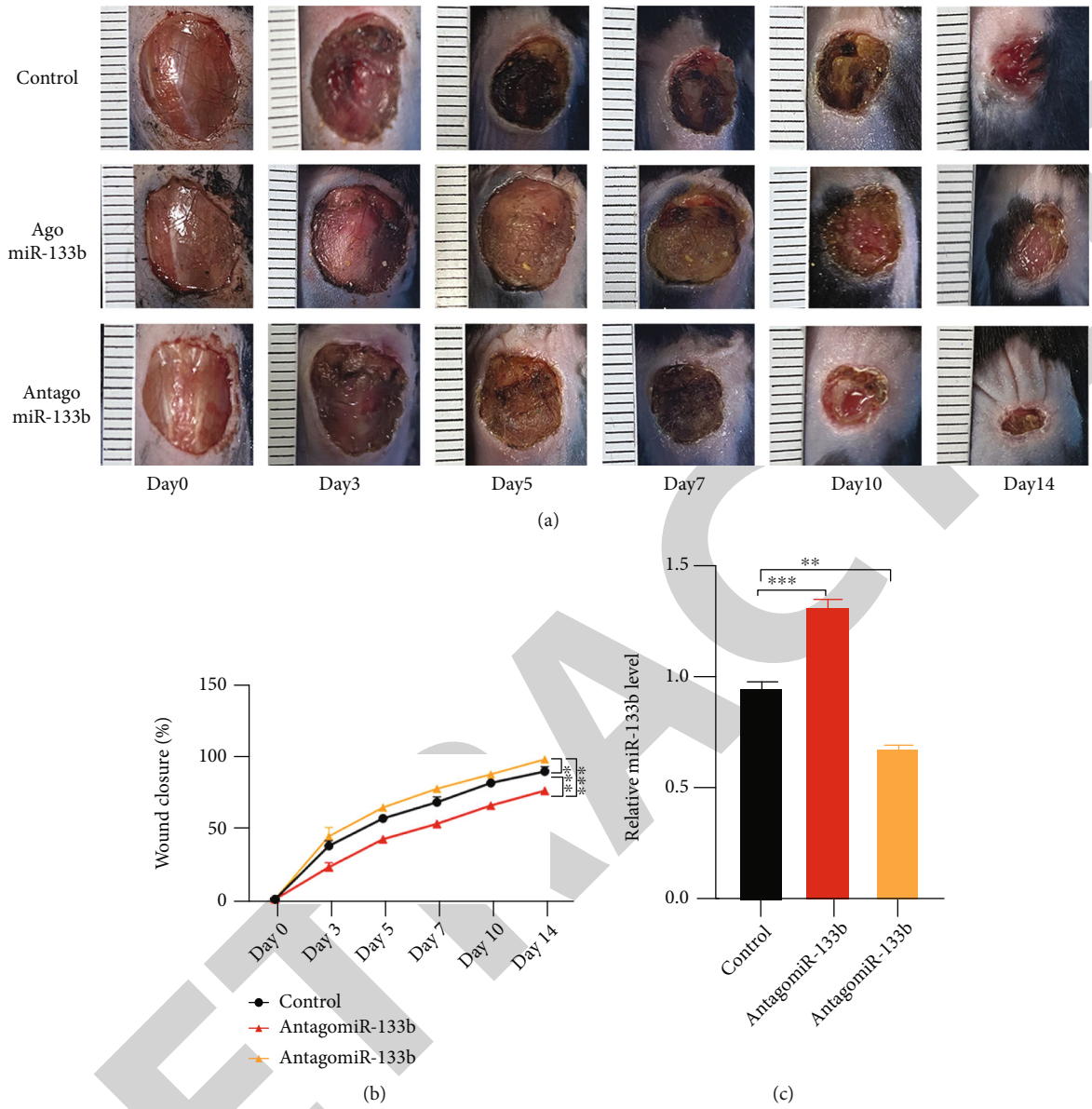

Figure 7: Administration of antagomiR-133b accelerates wound healing *in vivo*. (a) General condition of wounds after treatment with PBS (control), agomiR-133b, and antagomiR-133b. (b) Wound closure rates. (c) Expression of miR-133b in wound samples.

with antagomiR-133b (Figures 6(c) and 6(d)). Furthermore, HUVECs transfected with a miR-133b antagonist showed an improved migration ability (Figures 6(e) and 6(f)).

3.7. miR-133b Suppression Accelerates Wound Healing In Vivo. The effect of miR-133b inhibition on wound repair was assessed by injecting equal volumes of PBS (control group), agomiR-133b, or antagomiR-133b into the back wounds of control C57BL/6J mice and db/db mice. Wound closure analyses revealed that the antagomiR-133b group showed better wound healing than the other two groups (Figures 7(a) and 7(b)). miR-133b levels showed an obvious decrease in wounds only in mice treated with antagomiR-133b, suggesting that the enhancement of wound closure was indeed related to miR-133b suppression (Figure 7(c)).

Microarrays are widely used to investigate the pathogenic mechanisms mediating disease progression. Hence, they are extremely useful for functional genomic research [19, 20]. Studies have used microarrays to detect genetic contributors to diabetes-impaired wound healing. Consequently, several relevant genes such as *CD44*, *CCL5*, and *IL-6* [21, 22]—which play vital structural and functional roles in the regulation of diabetic wound progression—have been identified. Based on transcriptomic microarray data from DFUs and nondiabetic foot skin samples and subsequent DEG identification and GO and KEGG enrichment analyses, we identified *EGFR* as a potentially critical player in delayed wound healing in DFUs. After confirming that *EGFR* is downregulated in DFU patients, we performed thorough *in vitro* and *in vivo* experiments to provide

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confirmatory evidence showing that *EGFR* downregulation is a significant contributor to DFU progression.

Angiogenesis allows oxygen and nutrients to reach wound sites and is therefore vital for facilitating wound repair [23]. Endothelial cell dysfunction is closely linked to impaired angiogenesis, an important hallmark of diabetes and a key cause of impaired DFU healing [4, 24, 25]. EGFR signaling is involved in several biological processes and influences angiogenesis via the regulation of endothelial cell proliferation, apoptosis, and migration [26]. A previous study demonstrated that high-glucose levels impair EGFR signaling and attenuate *ex vivo* corneal epithelial wound healing [27]. Consistent with this finding, the present study showed that *EGFR* downregulation occurred in both high-glucose-treated HUVECs and skin wounds from diabetic mice.

miRNAs are noncoding, single-stranded RNAs that modulate gene expression. Interestingly, recent research has shown that miRNAs can be released by cells into the blood. These miRNAs can be taken up by other cells, leading to gene modulation in these cells [28]. Because endothelial vascular cells are exposed to large amounts of circulating miRNAs, they are especially susceptible to their regulatory effects. A recent study performed in a rat model of aortic balloon injury showed that endothelial progenitor cells release exosomes containing miR-21-5p, which—upon internalization by endothelial cells—mediate vascular repair by suppressing thrombospondin-1, an angiogenesis inhibitor [10]. Previous research has also shown that *EGFR* expression is regulated by several miRNAs in different tumors [29, 30]. For example, a study showed that miR-522-3p overexpression could mediate acquired resistance against EGFR-tyrosine kinase inhibitors in NSCLC [31]. Therefore, we searched for potential regulators of *EGFR* expression by screening for miRNAs that could bind to *EGFR* mRNA. Using several online predicting tools, we identified five candidates—miR-133b, miR-16, miR-223, miR-133bp, and miR-337. Given the recently reported link between miR-133b and *EGFR* in squamous cell carcinoma [32], in our study, we examined how miR-133b affects both *EGFR* expression and wound healing. We found that this miRNA has detrimental effects on both vascular endothelial cell function and wound repair. When a miR-133b agonist was transfected into HUVECs, we observed a significant reduction in their proliferation, migration, and tube-forming ability and an increase in their rate of apoptosis. Accordingly, we observed reduced wound perfusion and delayed wound healing after the treatment of wounds in mice with miR-133b mimics. It is worth noting that angiogenesis is not the only mechanism affected in DFU and that miR-133b can regulate other processes, such as inflammation and fibrosis [33, 34]. However, our findings indicate that miR-133b inhibition can restore *EGFR* expression and accelerate diabetes-impaired wound healing.

4. Conclusions

Given the limited success of current treatments for DFUs, novel strategies that consider the underlying angiopathy are urgently required. Our findings suggest that exposure to circulating miR-133b may lead to negative effects in endothelial cells by impairing EGFR signaling and contribute to delayed wound healing in diabetic patients. Therefore, the inhibition of miR-133b could represent a potential therapeutic strategy for promoting the healing of diabetic wounds.

Data Availability

Data are available on request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Haobo Zhong, Jin Qian, and Zhihong Xiao contributed equally to this work.

Acknowledgments

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Research Article

Exosomes Derived from Bone Mesenchymal Stem Cells Alleviate Compression-Induced Nucleus Pulposus Cell Apoptosis by Inhibiting Oxidative Stress

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Oxidative stress is relevant in compression-induced nucleus pulposus (NP) cell apoptosis and intervertebral disc (IVD) degeneration. Exosomes derived from bone mesenchymal stem cells (BMSCs-Exos) are key secretory products of MSCs, with important roles in tissue regeneration. This research is aimed at studying the protective impact of BMSCs-Exos on NP cell apoptosis caused by compression and investigating the underlying mechanisms. Our results indicated that we isolated BMSCs successfully. Exosomes were isolated from the BMSCs and found to alleviate the inhibitory effect that compression has on proliferation and viability in NP cells, decreasing the toxic effects of compression-induced NP cells. AnnexinV/PI double staining and TUNEL assays indicated that the BMSCs-Exos reduced compression-induced apoptosis. In addition, our research found that BMSCs-Exos suppressed compression-mediated NP oxidative stress by detecting the ROS and malondialdehyde level. Furthermore, BMSCs-Exos increased the mitochondrial membrane potential and alleviated compression-induced mitochondrial damage. These results indicate that BMSCs-Exos alleviate compression-mediated NP apoptosis by suppressing oxidative stress, which may provide a promising cell-free therapy for treating IVD degeneration.

1. Introduction

Low back pain (LBP) has emerged as a health issue affecting quality of life and exerting a financial burden on patients and healthcare systems. IVD degeneration is the principal cause of LBP [1, 2]. Therapy of IVD degeneration typically entails conservative treatment or spinal operation to reduce the patient's pain [3, 4]. These treatments are, however, symptomatic and do not cure IVD degeneration. Many complicated factors, including aging and mechanical stress are associated with IVD degeneration. Excessive mechanical loading is regarded as a key cause of IVD degeneration [5, 6]. However, the mechanisms of IVD degeneration have not been completely elucidated.

Oxidative stress is widely considered to be a crucial mechanism of IVD degeneration. Recent studies reported that IVD degeneration is strongly associated with oxidative stress [7, 8]. Although ROS production is important for maintaining intracellular redox homeostasis, excessive ROS production can cause oxidative stress damage leading to cell damage and even death [8, 9]. Growing evidence has revealed that excessive oxidative stress leads to IVD cellular apoptosis, resulting in a reduction in the number of IVD cells. These changes contribute to IVD degeneration [10,

11]. Recent studies have reported that hydrogen peroxide can cause mitochondrial dysfunction and even NP cell death by enhancing oxidative stress, which resulted in IVD degeneration [12]. Previous research also reported that compression induces NP cell apoptosis and decreases mitochondrial membrane potential (MMP) by oxidative stress, leading to IVD degeneration [13]. Based on these studies, searching for an effective means of decreasing the NP cell damage caused by oxidative stress may offer a therapeutic target to treat IVD degeneration.

Stem cell therapy is regarded as an important way for treating several diseases [14, 15]. Interestingly, evidence from prior studies indicated that stem cells can serve their tissue repair function through paracrine exosomes. Exosomes, microvesicles with a diameter between 30 and 150 nm, can deliver growth factors, and proteins to target cells and exert their function [16, 17]. Exosomes have many advantages compared to MSCs, including greater stability and low immunogenicity [18–20]. A recent study reported that MSCs-Exos inhibit TNF-α-induced NP apoptosis and restore IVD degeneration [21]. Besides, studies showed that MSCs-Exos ameliorate H_2O_2-induced NP cell damage and repair intervertebral disc degeneration via inhibition of oxidative stress [22]. However, the impact of MSCs-Exos on NP death caused by compression remains unclear.

In this research, we isolated BMSCs-Exos and further investigated the protective role of BMSCs-Exos on NP apoptosis induced by compression. Besides, we assessed the impact of oxidative stress in BMSCs-Exos against NP cell apoptosis by measuring ROS level and assessing the function of mitochondria.

2. Material and Methods

2.1. Isolation and Identification of BMSCs. This study was approved by the Clinical Research Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. According to our previous research, SD rats were isolated BMSCs [16, 23]. Briefly, the bone marrow of the femur and tibia was washed with a syringe. BMSCs were then incubated in a fresh medium containing 10% depleted exosomes of FBS. After 24 hours, fresh culture media was added. BMSCs were incubated with anti-CD73, CD90, CD34, and CD45 solutions to detect the specific marker. Flow cytometry (Becton Dickinson, USA) was used for testing the labeled cells. The multilineage differentiative capacity was assessed with alizarin red, oil red O, or alcian blue (Cyagen, USA). The results were observed with a microscope (Olympus, Japan).

2.2. Isolation and Identification of Exosomes. Ultracentrifugation was applied to isolate and extract BMSCs-Exos. Briefly, the supernatant of BMSCs was collected and centrifuged at 2000 g for 30 minutes. The conditioned medium was centrifuged at 12000 g for 45 minutes and then centrifuged at 100,000 g for 90 minutes. Subsequently, sediment obtained was resuspended with PBS and stored at -80°C. For the validation of exosomes, transmission electron microscopy (TEM) was viewed BMSCs-Exos shape. The size distribution of BMSCs-Exos was tested by nanoparticle tracking analysis (NTA). Specific markers of BMSCs-Exos (CD9, CD63) were detected by western blotting.

2.3. Exosome Uptake. A BMSCs-Exos uptake assay was conducted using PKH26 red dye (Sigma-Aldrich, USA). Briefly, BMSCs-Exos were marked with PKH26 according to the instruction book. The NP cells were incubated with PKH26-labeled exosomes. Then, cells were fixed with 4% paraformaldehyde. FITC phalloidin (Solarbio, China) was used to observed labeled the cytoskeleton of NP cells. The NP nuclei were stained with Hoechst 33342. The cells were observed with confocal microscopy (Nikon A1, Japan).

2.4. Culture and Treatment of NP Cells. NP cells were obtained and cultured from SD rats (200-250 g) as we described previously [12, 13, 24]. Briefly, after anesthesia with intraperitoneal injection of chloride hydrate, the IVD of rats were harvested immediately. After disinfection, the NP tissue was collected from rat lumbar discs. Then, the obtained NP tissue was digested with 0.25% type II collagenase (Biosharp, China) for 15 minutes. The NP tissue was centrifuged and cultured in DMEM/F-12 (Gibco, USA) containing 10% FBS (Gibco, USA). The NP cells of second generation were used for further experiments. To test the impact of BMSCs-Exos on NP cells, the cells were cultured in a pressure apparatus to simulate mechanical loading conditions of IVD as we described previously [13, 24, 25]. Briefly, the cells received different concentrations of BMSCs-Exos (20, 50, or 100 μg/ml) and received 1.0 MPa compression treatment for 36 hours. Then, the NP cells were used for further experiments. A control group (con) was cultured under the same conditions without compression.

2.5. CCK-8 Assay. Cell viability was tested by Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the instruction book. In brief, NP cells were cultured in 96-well plates and received different treatments for 36 h, including BMSCs-Exos and compression. After treatment, 100 ml CCK-8 solution containing 90 ml DMEM/F-12 was added in 96-well plates. The cells were hatched at 37°C in the dark. Then, the absorbance at 450 nm was measured by a microplate reader (BioTek, USA).

2.6. EdU Incorporation Assay. Cell proliferation was tested by a EdU assay (RiboBio, China) as we used previously [26]. In brief, after receiving different treatments for 36 h, including BMSCs-Exos and compression, the NP cells were assessed with an EdU assay according to the instruction book. NP cells were washed with PBS. Finally, the results of EdU assay were observed with a microscope (Olympus, Japan).

2.7. Live/Dead Assay. The live/dead effect of BMSCs-Exos on NP cells induced by compression was assessed by the Calcein-AM/PI probe (Dojindo, Japan). Briefly, after receiving different treatments including BMSCs-Exos and compression for 36 h, the cells were treated with Calcein-AM and PI staining for 20 minutes according to the instruction book.

After being washed, the live/dead cells were observed with a microscope (Olympus, Japan).

2.8. Lactate Dehydrogenase Release Assay. Lactate dehydrogenase (LDH) release tested the impact of BMSCs-Exos on NP cells induced by compression. After NP cells received different treatments for 36 h, including BMSCs-Exos and compression, the release of LDH from the cells was detected by the LDH assay according to the instruction book. Absorbance at 490 nm was measured by a microplate reader (BioTek, USA).

2.9. Annexin V/PI Staining. The Annexin V-FITC/PI apoptosis assay (KeyGen Biotech, China) was detected the apoptotic impact of BMSCs-Exos on NP cells induced by compression according to the instruction book. In brief, after receiving different treatments including BMSCs-Exos and compression for 36 h, the NP cells were digested by trypsinization and collected. The cells were resuspended in a binding buffer. Subsequently, Annexin V and PI staining were added in NP cells for 15 minutes. Cells were tested by flow cytometry (Becton Dickinson, USA).

2.10. TUNEL Staining. TUNEL assays tested the apoptosis of NP cells according to the instruction book. Briefly, after being received different treatments for 36 h, including BMSCs-Exos and compression, the cells were fixed with 4% paraformaldehyde. Cells were washed and were permeabilized with TritonX-100 for 10 minutes. Then, cells were treated with TUNEL staining (Roche, Germany). The cells were stained with DAPI. The results were observed with a microscope (Olympus, Japan).

2.11. Measurement of Cellular ROS. DCFH-DA assay (Nanjing Jiancheng, China) tested the intracellular ROS level according to the instruction book. In brief, after being treated with compression and BMSCs-Exos for 36 h, NP cells were digested by trypsinization and collected. Subsequently, cells were incubated with DCFH-DA at 37°C. Cells were tested by flow cytometry (Becton Dickinson, USA).

2.12. MDA Assay. The MDA concentration of cells was tested by a Lipid Peroxidation MDA Assay (Beyotime). Briefly, after NP cells received different treatments for 36 h, including compression and BMSCs-Exos, the cells were lysed and then centrifuged at. MDA solution was added in the supernatant according to the instruction book. Absorbance at 532 nm was tested by a microplate reader (BioTek, USA).

2.13. JC-1 Staining. JC-1 staining assay (Beyotime, China) tested NP cell mitochondrial membrane potential (MMP) according to the instruction book. Briefly, after being treated with compression and BMSCs-Exos for 36 h, NP cells were digested and collected. Subsequently, cells were stained with a JC-1 fluorescent probe at 37°C for 20 minutes. Cells were centrifuged and resuspended in a staining buffer. Cells were tested by flow cytometry (Becton Dickinson, USA). Besides, MMP was also tested by a fluorescence microscope (Olympus, Japan).

2.14. Mitotracker Staining. The mitochondria of NP cells were detected with mitotracker staining (ThermoFisher, USA) according to the instruction book. Briefly, after receiving different treatments including compression and BMSCs-Exos for 36 h, NP cells were washed twice and incubated with a mitotracker staining probe. Subsequently, the cells were counterstained with Hoechst 33342. After being washed, cells were observed in the dark by a microscope (Olympus, Japan).

2.15. Statistical Analysis. GraphPad Prism (GraphPad Software Inc, USA) was used for statistical analysis. Data were expressed as mean ± standard deviation (SD) from three independent experiments. Differences between groups were analyzed by Student's t-test and the ANOVA test. Differences in more than two groups were analyzed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of BMSCs. BMSCs were isolated and cultured from rat bone marrow. Flow cytometry was used for surface marker detection on the BMSCs, demonstrating that the isolated cells highly expressed specificity surface markers of stem cells, including CD73 and CD90. The positive rates of the cells were greater than 95%. Our results indicated that these cells lowly expressed CD34 and CD45 (Figures 1(a) and 1(b)). The multilineage differentiation of these cells was detected using a BMSCs-osteogenic, adipogenic, and chondrogenic differentiation medium. Our results revealed that the cells with osteogenic induction formed multiple calcium deposits, visualized with alizarin red staining. Oil droplets were formed in the BMSCs with oil red O staining after treating the cells with an adipogenic differentiation medium. After treatment with a chondrogenic differentiation medium, the cells produced acidic mucopolysaccharide visualized with alcian blue (Figure 1(c)). Together, our results displayed that the cells isolated met the criteria of MSCs as described by International Society for CellularTherapy.

3.2. Characterization of BMSCs-Exos. Ultracentrifugation was used to isolate and extract BMSCs-Exos from the medium of BMSCs to test the impact of BMSCs-Exos on NP cells. The exosomes isolated displayed spherical shape and membrane vesicles from the result of TEM (Figure 2(a)). The size distribution of exosomes was detected by NTA, identifying that the exosomes mainly ranged from 30 to 150 nm (Figure 2(b)). Western blotting was tested specificity markers, showing that the exosomes derived from BMSCs expressed specificity markers of exosomes, such as CD9 and CD63 (Figure 2(c)). These data showed that the isolated exosomes possessed exosomal characteristics. In addition, PKH26 probing assessed the exosomal uptake by NP cells. After labeling the exosomes with PKH26, confocal microscopy revealed that the BMSCs-Exos were endocytosed by NP cells (Figure 2(d)).

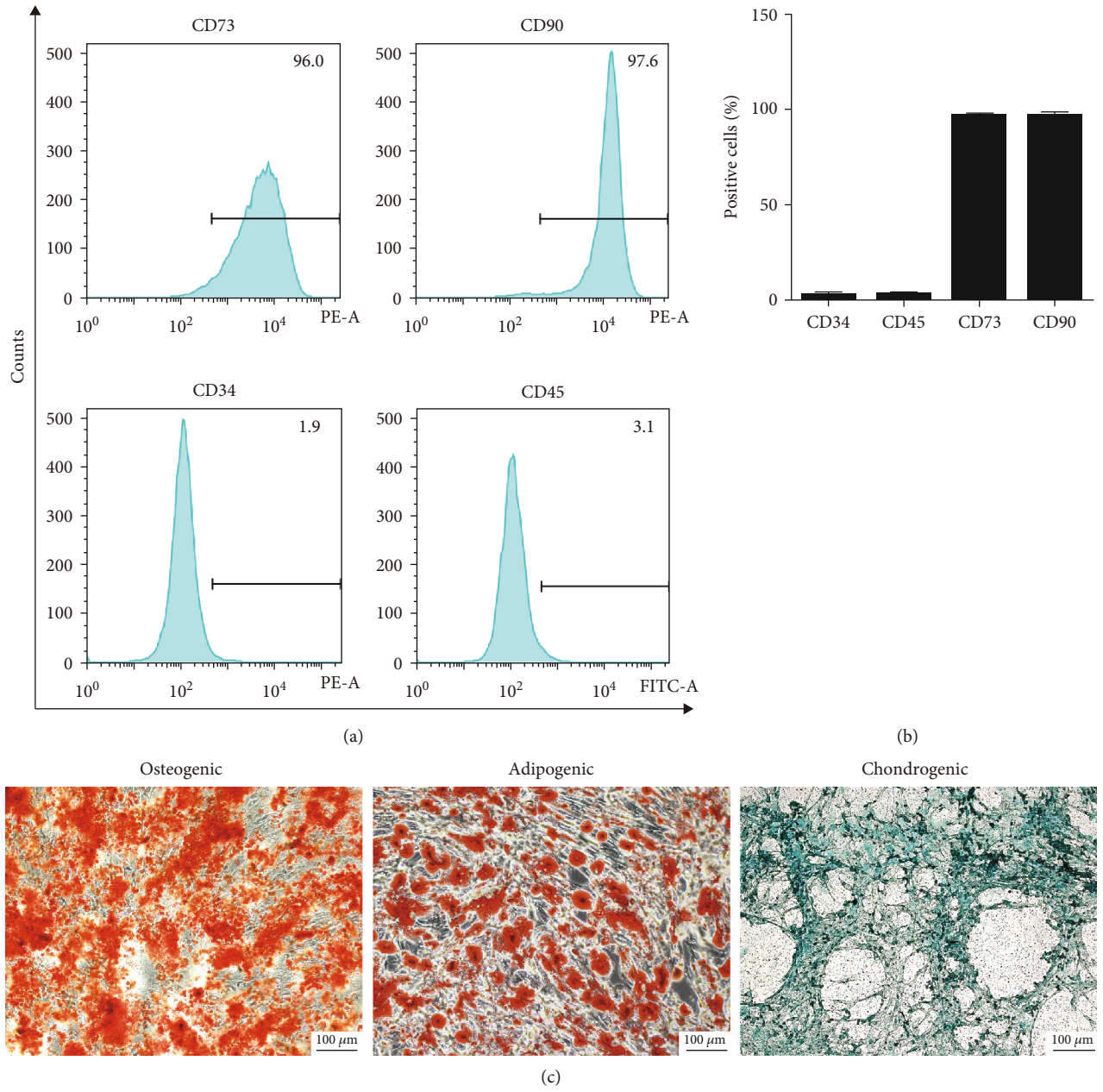


Figure 1: Identification of BMSCs. (a) Flow cytometry tested the CD34, CD45, CD73, and CD90 surface markers on the BMSCs. (b) Quantification analysis of positive cells in BMSCs. (c) Alizarin red, oil red O, and alcian blue staining tested osteogenic, adipogenic, and chondrogenic differentiation of BMSCs, respectively. Scale bar: 100 μm.

3.3. BMSCs-Exos Alleviate Compression-Induced Cytotoxicity in NP Cells. CCK-8 assay tested the impact of BMSCs-Exos on NP cell viability. The cell viability was significantly decreased by compression. BMSCs-Exos significantly attenuated the suppressive impact of compression on NP cell viability. The protective impact of BMSCs-Exos was greatest at a dose of 100 μg/ml; therefore, 100 μg/ml BMSCs-Exos was employed in subsequent experiments (Figure 3(a)). The impact of BMSCs-Exos on NP cell proliferation was tested by an EdU incorporation assay. The results demonstrated that the EdU-positive cells in BMSCs-Exos groups was greater than that in the compression groups alone (Figures 3(b) and 3(c)). To test the protective impact of BMSCs-Exos on NP cells, Calcein-AM/PI probing tested the cytotoxicity effect of BMSCs-Exos or compression on NP cells. The results showed that live cells (green fluorescence) were greater in the BMSCs-Exos groups while dead cells (red fluorescence) were higher in the compression group (Figure 3(d)). Subsequently, LDH assay tested the toxicity effect of NP cells. We found that the release of LDH was increased in the compression group, while the release of LDH was alleviated by BMSCs-Exos compared with the compression group (Figure 3(e)). Overall, BMSCs-Exos relieved compression-induced NP cell cytotoxicity.

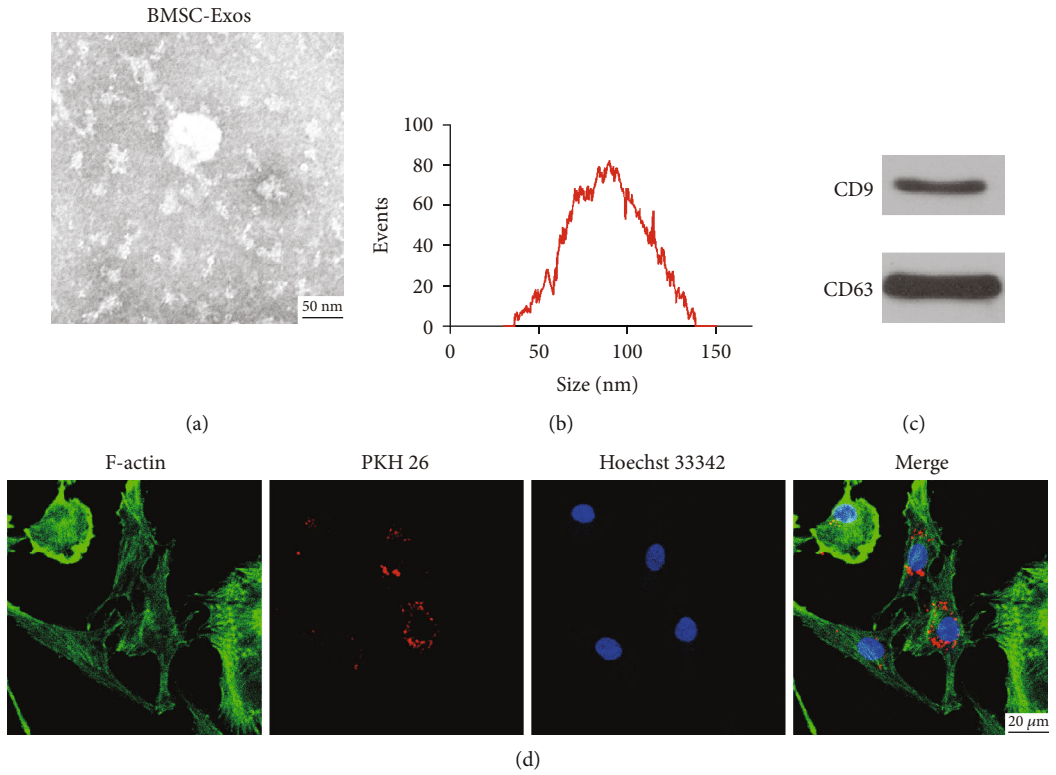


Figure 2: Characterization of BMSC-Exos. (a) The exosome morphology was observed by TEM. Scale bar: 50 nm. (b) Size distribution of BMSC-Exos was examined with NTA. (c) The exosomal marker proteins CD9 and CD63 of BMSC-Exos were tested by western blotting. (d) The assay of BMSC-Exos uptake by NP cells was observed by laser scanning confocal microscopy. The exosomes, cytoskeleton, and cell nucleus are displayed red, green, and blue, respectively. Scale bar: 20 μm.

3.4. BMSCs-Exos Reduced Compression-Caused NP Cell Apoptosis. To test the impact of BMSCs-Exos on cell apoptosis caused by compression, Annexin V/PI staining detected NP cell apoptosis. Flow cytometry revealed that compression significantly increased NP cell apoptosis. BMSCs-Exos significantly alleviated NP cell apoptosis caused by compression (Figures 4(a) and 4(b)). In addition, we also evaluated the NP cell apoptosis by TUNEL assays. We found that the number of TUNEL-positive cells (green fluorescence) in compression groups was increased compared with the control groups, while BMSCs-Exos decreased the number of TUNEL-positive cells induced by compression (Figure 4(c)). Our research indicated that BMSCs-Exos reduced compression-induced cell apoptosis in NP cells.

3.5. BMSCs-Exos Decreased Compression-Caused Oxidative Stress in NP Cells. Growing evidences have revealed that mitochondrial dysfunction leads to cell oxidative stress and apoptosis. To explore the impact of BMSCs-Exos on NP cell oxidative stress caused by compression, DCFH-DA fluorescent probes examined ROS level. Flow cytometry revealed that compression promoted NP cell ROS production and caused oxidative stress, while ROS level of NP cells in BMSCs-Exos groups was markedly decreased compared with compression groups alone (Figures 5(a) and 5(b)). To further test the suppressive impact of BMSCs-Exos on oxidative stress, we used MDA assay to further investigate the oxidative stress level. We found that compression significantly increased the MDA content in NP cells, while BMSCs-Exos observably decreased the MDA level induced by compression (Figure 5(c)). Our research showed that BMSCs-Exos decreased compression-induced oxidative stress in NP cells.

3.6. BMSCs-Exos Protected against Compression-Caused Mitochondrial Damage in NP Cells. To study the protective impact of BMSCs-Exos on mitochondrial damage caused by compression in NP cells, flow cytometric analysis was tested MMP by JC-1 staining. The results displayed that compression significantly decreased the red/green ratio in NP cells compared with the control group, and the ratio of red to green in the BMSCs-Exos group was significantly increased than that in the control group (Figures 6(a) and 6(b)). In addition, we observe the protective effects of BMSCs-Exos on MMP by a fluorescence microscope; our results demonstrated that normal NP cells of the control group mainly showed red fluorescence. However, the cells in the compression group exhibited more green fluorescence while faint red fluorescence. BMSCs-Exos improve red fluorescence inhibited by compression (Figure 6(c)). Besides, we further used mitotracker staining to show the impact of BMSCs-Exos on NP cell mitochondria. The results of the fluorescence microscope revealed that compression obviously decreased intracellular mitochondria compared with the control group, while BMSCs-Exos protected against compression-induced intracellular mitochondria (Figure 6(d)). Those results demonstrated

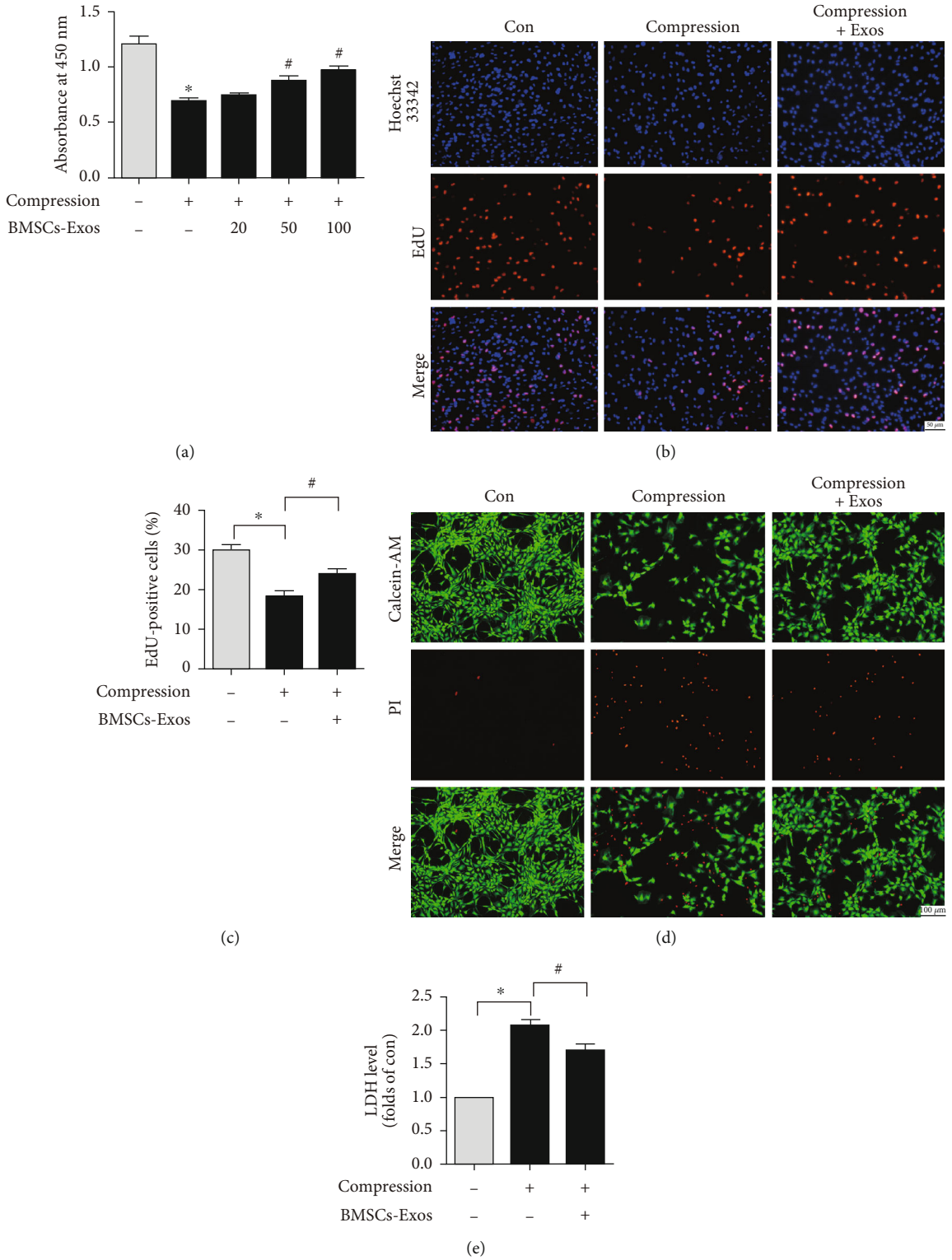


Figure 3: BMSC-Exos alleviate compression-caused cytotoxicity in NP cells. (a) CCK-8 assay tested the impact of BMSC-Exos (0, 20, 50, and 100 μg/ml) and compression on NP cell viability. (b) The NP cell proliferation was tested by an EdU incorporation assay. The EdU-positive cells displayed red fluorescence. Scale bar: 50 nm. (c) Quantification analysis percentage of EdU-positive cells. (d) Calcein-AM/PI staining tested the cytotoxicity effect of BMSC-Exos on NP cells caused by compression. Scale bar: 100 nm. (e) The released result of LDH in NP cells. Data are expressed as the mean ± SD from three independent experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the compression group.

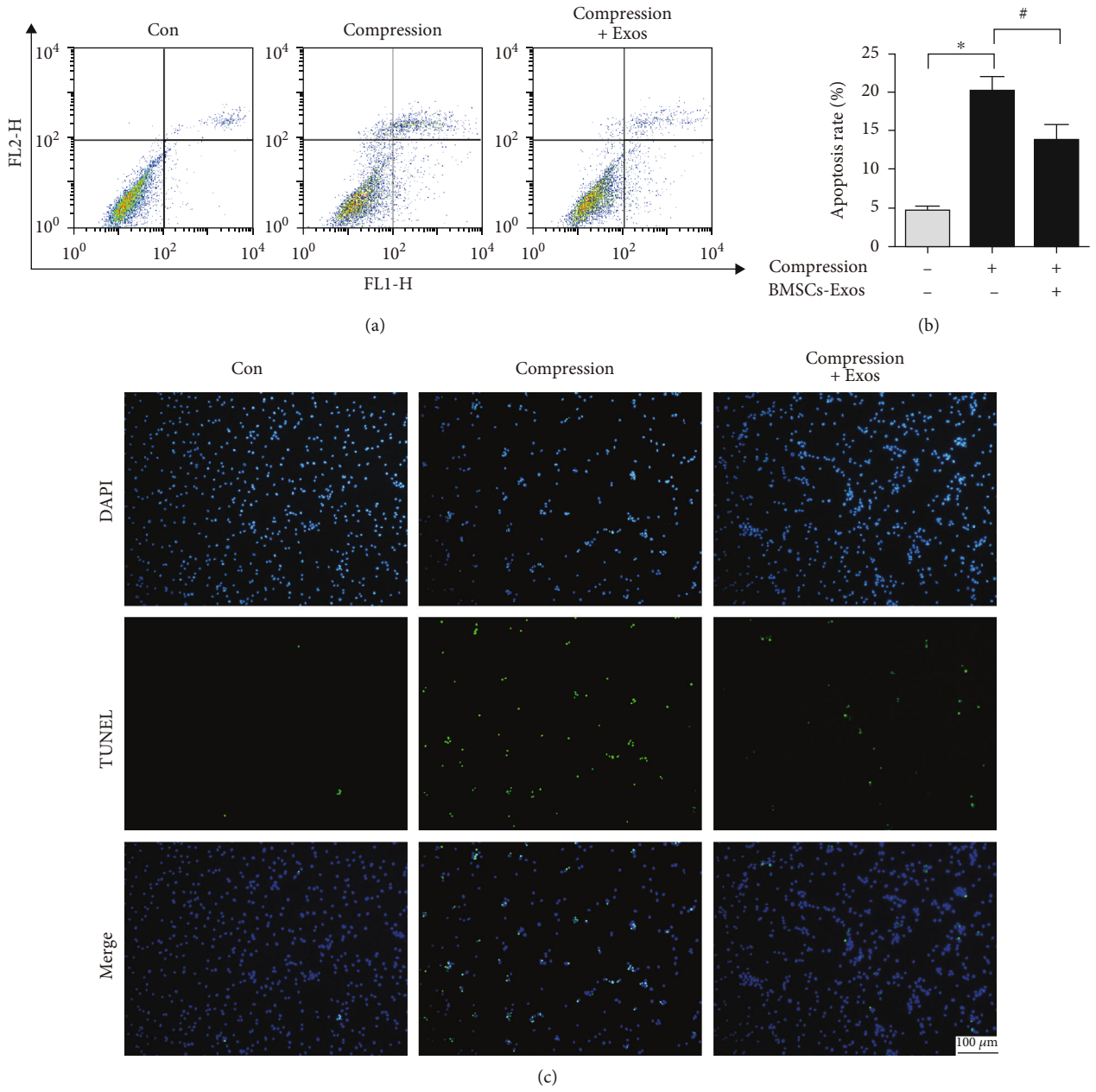


Figure 4: BMSC-Exos reduced compression-caused apoptosis in NP cells. (a) Annexin V/PI staining tested NP cell apoptosis by flow cytometry. (b) Quantification analysis of NP cell apoptosis. (c) The TUNEL staining observed NP cell apoptotic changes. Scale bar: 100 nm. Data are expressed as the mean ± SD from three independent experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the compression group.

that BMSCs-Exos protected against compression-induced mitochondrial damage in NP cells.

4. Discussion

In this study, we firstly detected whether BMSCs-Exos protected NP cell apoptosis caused by compression and the possible molecular mechanisms. We successfully isolated BMSCs-Exos and found that BMSCs-Exos reduced the suppressive impact of compression on viability and proliferation of NP cells. Our research revealed that BMSCs-Exos reduced NP cell cytotoxicity caused by compression. BMSCs-Exos also relieved compression-mediated NP cell apoptosis by inhibiting oxidative stress and mitochondrial damage.

Low back pain is one of the most prevalent bone diseases around the world. IVD degeneration is an essential reason for LBP. Growing evidence has revealed that mechanical compression is a principal cause of IVD degeneration [27, 28]. IVDs experience various mechanical loads during daily activities. Recent studies have displayed that mechanical loading reduced the activity and number of IVD cells, leading to IVD degeneration [29]. Excessive IVD cell death under a compressed microenvironment makes it difficult to maintain IVD activity and number [30]. The unfavorable

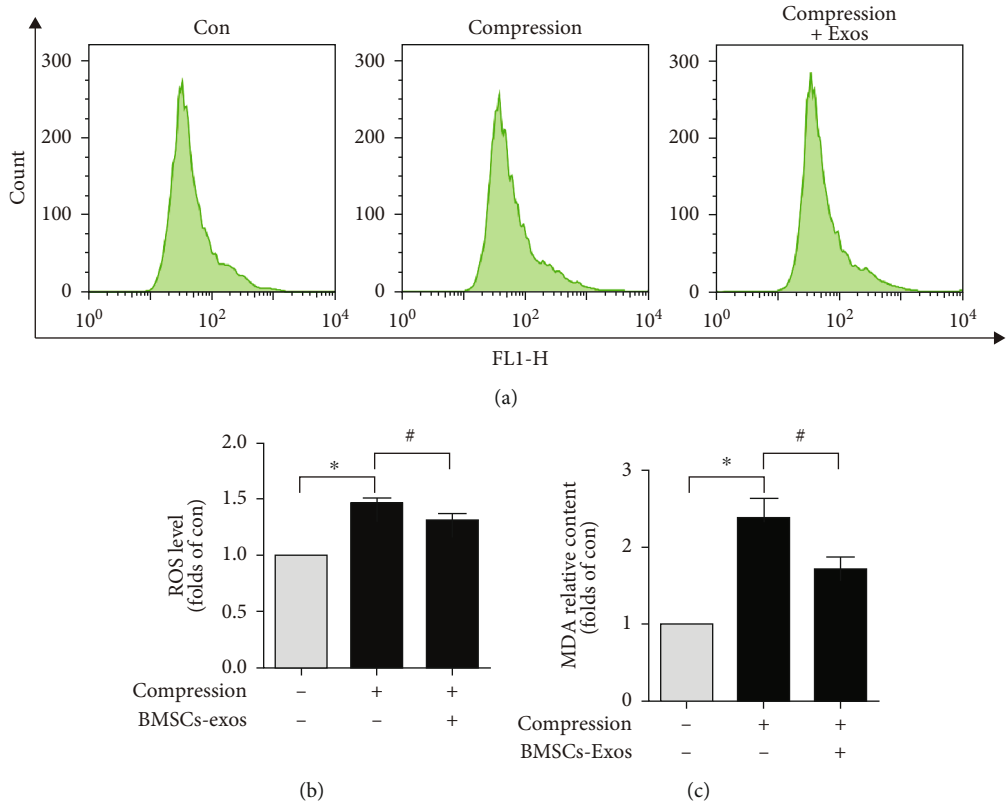


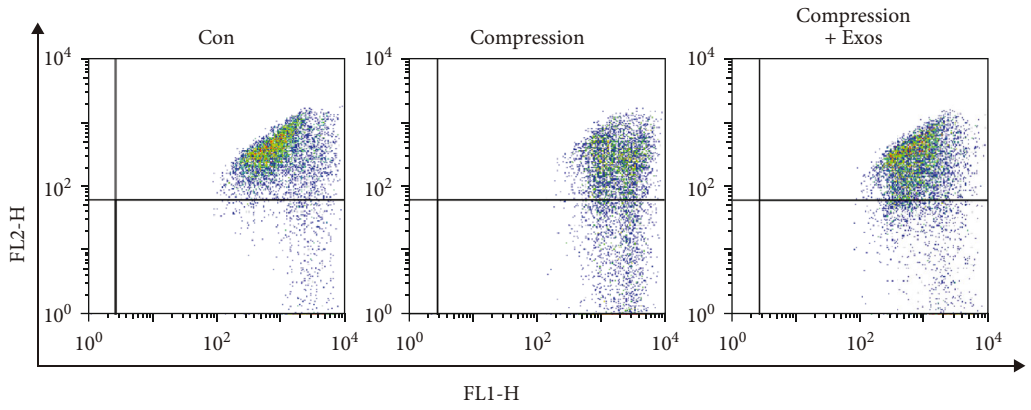
Figure 5: BMSC-Exos decreased compression-induced oxidative stress in NP cells. (a) DCFH-DA probes tested NP cell ROS level by flow cytometry. (b) Quantification analysis of ROS level in NP cells. (c) MDA assay was tested MDA levels. Data are presented as the mean ± SD from three independent experiments. $^*P < 0.05$ versus the control group; $^\#P < 0.05$ versus the compression group.

factors can result in IVD degeneration. Huang et al. showed that compression induces senescence of NP cells and contributes to IVD degeneration [31]. Based on this research, maintenance of the number and viability of IVD cells is considered an important therapeutic strategy for IVD degeneration.

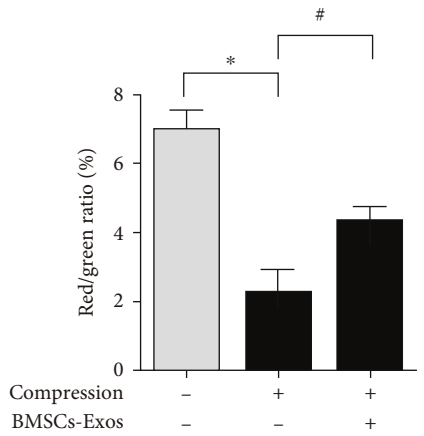
Exosomes secreted by MSCs are critical secretion of MSCs which can mediate cell communication between MSCs and other cells. Recent studies displayed that exosomes are a treatment modality that may offer a promising therapeutic strategy for tissue regeneration [32, 33]. Exosomes have received extensive attention because they were reported to possess functional proteins and miRNAs that have many positive impacts in mediating cellular function and treating a variety of diseases [16, 34, 35]. Recent studies indicated that MSCs-Exos protect endplate chondrocytes against death and offer a potential strategy for IVD degeneration [36]. Zhang et al. reported that MSC-derived Exos ameliorate LPS-induced cell pyroptosis, treating IVD degeneration [37]. In our study, we found that BMSCs-Exos alleviated the suppressive impact of compression on NP cell viability. BMSCs-Exos also promoted NP cell proliferation inhibited by compression. In addition, our results displayed that BMSCs-Exos alleviated cytotoxicity caused by compression in NP cells.

Cell apoptosis plays a crucial role in the clearance of damaged or nonessential cells. Many studies recently reported that apoptosis is considered a crucial way of IVD cell death and plays an essential role in IVD degeneration process [26, 38]. Our previous research showed that compression increased NP cell apoptosis, causing IVD degeneration [13]. Lin et al. showed that edaravone could ameliorate NP cell cytotoxicity and apoptosis caused by compression, highlighting a potential treatment of IVD degeneration [24]. Recent research has reported that regulating TIGAR improved compression-induced NP cell apoptosis, promoting IVD degeneration repair [25]. Interestingly, Zhu et al. reported that MSC-derived exosomes ameliorate interleukin-1β-induced NP cell apoptosis. They support that MSC-derived exosomes have therapeutic potential for IVD degeneration diseases [39]. In our research, our results showed that BMSCs-Exos alleviate compression-induced cell apoptosis in NP cells.

Multiple studies displayed that apoptotic signals trigger the mitochondrial dysfunction resulting in an increase in ROS production and establishment of an oxidative stress environment. Oxidative stress caused cell death in IVD degeneration [26, 28]. Recent researchers reported that excess ROS caused mitochondria and DNA damage, which leads to cell injury and even death [24, 40]. Recent research has reported that CsA relieved NP stem cell apoptosis mediated by compression via alleviating oxidative stress and mitochondrial dysfunction, which suggested that CsA is a potential way for IVD degeneration [40]. Chen et al. reported oxidative stress played a vital role in NP cell apoptosis caused by compression. They support that regulation



(a)



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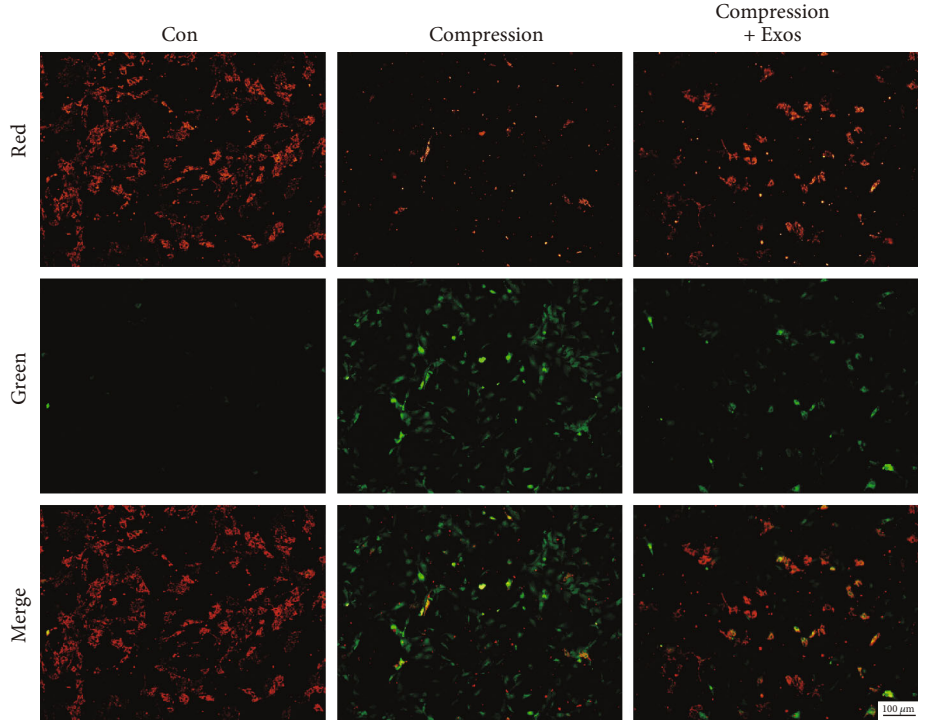

(c)

Figure 6: Continued.

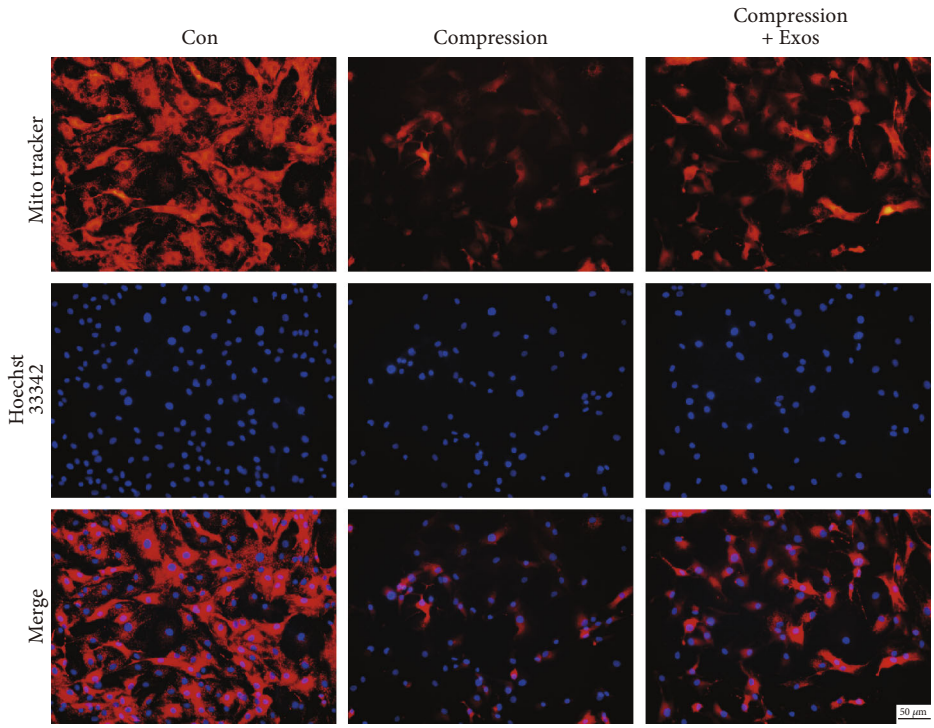

Figure 6: BMSC-Exos protected against compression-induced mitochondrial damage in NP cells. (a) JC-1 staining tested MMP by flow cytometry. (b) Quantification analysis of JC-1 assay. (c) JC-1 staining was tested under a fluorescence microscope in NP cells. Scale bar: 100 nm. (d) Mitotracker staining tested cell mitochondria. Scale bar: 50 nm. Data are presented as the mean ± SD from three independent experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the compression group.

of cell death such as necroptosis and apoptosis may contribute to NP cell survival and repair IVD degeneration [28]. In addition, exosomes from adipose stem cells alleviated oxidative stress in macrophages to promote tissue repair and regeneration [41]. Exosomes derived from MSCs ameliorate H_2O_2-caused mitochondrial dysfunction and prevent the progression of IVD degeneration by inhibiting inflammation [22]. In our study, we found BMSCs-Exos decreased compression-caused NP cell oxidative stress by decreasing ROS production. BMSCs-Exos also protected against compression-decreased MMP and induced mitochondrial damage in NP cells.

There are several limitations in our research. First, all of our experiments were performed in *vitro*, and the results may not necessarily be indicative of what occurs in *vivo*. Second, this study focused on the protective impact of BMSCs-Exos on NP cell apoptosis caused by compression through inhibition of oxidative stress. We will investigate how BMSCs-Exos exert their protective effect in compression-induced oxidative stress in the future.

5. Conclusions

In this study, we successfully isolated exosomes from BMSCs and found that BMSCs-Exos alleviate NP cell viability caused by compression. The results indicated that BMSCs-Exos inhibited compression-induced cell cytotoxicity and apoptosis. The underlying protective mechanism of BMSCs-Exos on NP cell apoptosis-induced compression was linked to NP cell oxidative stress. Taken together, these findings may provide a promising cell-free therapy for treating IVD degeneration.

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 competing interests.

Authors' Contributions

YQH, RYT, and LC performed the experiments. ZL, HX, and HL analyzed the results. YQH and LFW wrote the manuscript. YQH, AP, and HL revised the paper. HL, LMX, and GHL reviewed the manuscript. All authors approved the final manuscript. Yiqiang Hu, Ranyang Tao, and Linfang Wang contributed equally to this work.

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Retraction

Retracted: Periodontitis Exacerbates Benign Prostatic Hyperplasia through Regulation of Oxidative Stress and Inflammation

Oxidative Medicine and Cellular Longevity

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope
(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
(4) Inappropriate citations
(5) Incoherent, meaningless and/or irrelevant content included in the article
(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] C. Fang, L. Wu, M. Zhao et al., "Periodontitis Exacerbates Benign Prostatic Hyperplasia through Regulation of Oxidative Stress and Inflammation," *Oxidative Medicine and Cellular Longevity*, vol. 2021, Article ID 2094665, 11 pages, 2021.

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Research Article

Periodontitis Exacerbates Benign Prostatic Hyperplasia through Regulation of Oxidative Stress and Inflammation

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Epidemiological studies demonstrate that men with periodontitis are also susceptible to benign prostatic hyperplasia (BPH) and that periodontal treatment can improve the prostatic symptom. However, molecular links of this relationship are largely unknown. The goal of the current study was to elucidate the effects of experimental periodontitis on the hyperplasia of prostate and whether oxidative stress and inflammation participated in this process. For this purpose, ligature-induced periodontitis, testosterone-induced BPH, and the composite models in rats were established. Four weeks later, all the rats were sacrificed and the following items were measured: alveolar bone loss and histological examination of periodontal tissues were taken to assess the establishment of periodontitis model, prostate index and histological examination of prostate tissues were taken to test the establishment of the BPH model, inflammatory cytokines in plasma were assessed, and Bax/Bcl-2 proteins related to cell apoptosis were analyzed via western blot analysis. To further investigate whether oxidative stress participates in the aggravation of BPH, *in vitro* models were also conducted to measure the production of intracellular reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) concentration. We found that simultaneous periodontitis and BPH synergistically aggravated prostate histological changes, significantly increased Ki67 proliferation, and reduced apoptosis in rat prostate tissues. Also, our results showed that periodontal ligation induced increased Bcl-2 protein expression, whereas Bax expression was decreased in BPH rats than in normal rats. Compared with the control group, periodontitis and BPH both significantly enhanced inflammatory cytokine levels of TNF-α, IL-6, IL-1β, and CRP. Furthermore, *Porphyromonas gingivalis* lipopolysaccharide induced enhanced generation of intracellular expression of ROS and H_2O_2 in BPH-1 cells. Our experimental evidence demonstrated that periodontitis might promote BPH development through regulation of oxidative stress and inflammatory process, thus providing new strategies for prevention and treatment of BPH.

1. Introduction

Benign prostatic hyperplasia (BPH) is a common age-related proliferative disease in males, which is characterized by increased number of epithelial and stromal cells in the periurethral area of the prostate [1, 2]. The prevalence of BPH increases from 25% in men aged 40 to 49 years to more than 80% in men aged 70 to 79 years [3]. Periodontitis is another prevalent age-related inflammatory disorder that affects the teeth's supportive tissues, which eventually contributing to tooth loss and systemic inflammation [4]. It is reported that populations aged 55 to 65 years are at the highest risk of developing severe periodontitis [5]. Epidemiological studies have demonstrated that periodontitis obviously raised the risk of BPH after adjusting for confounding factors [6, 7]. In recent years, links between periodontal disease and prostatic disease have gained increasing attention since both diseases share several common risk factors, such as age, metabolic disorders, and psychological factors [8, 9]. We have proposed an oral-prostate axis hypothesis in which

periodontitis may act on prostatic disease in a direct way through distant dissemination of oral bacteria (i.e., *Porphyromonas gingivalis*) and/or an indirect way through systemic inflammation; however, the precise mechanisms underlying these relationships are poorly understood [8, 10].

Numerous studies have demonstrated that oxidative stress is involved in the development of both periodontal and prostatic diseases [11, 12]. Oxidative stress is a situation when the oxidation system and antioxidant system is unbalanced with overproduction of reactive oxygen species (ROS) and a comparative deficiency of antioxidants [13]. Excessive accumulation of ROS can induce DNA damage, lipid peroxidation, and protein modification, subsequently causing cellular dysfunction and tissue damage [11, 14]. As one of the main sources of intracellular ROS, hydrogen peroxide (H_2O_2) is broadly used as an oxidative stressor in the *in vitro* models. Oxidative stress has been highly associated with the onset of several inflammatory pathologies occurring in elderly people, including periodontitis, osteoporosis, and metabolic disorders [12, 15, 16]. Also, clinical and experimental studies have a confirmed oxidative stress as a key contributor to the pathogenesis of BPH [17–20]. Moreover, compensatory cellular proliferation may be triggered by oxidative stress, which eventually leads to the growth of hyperplastic prostate tissue, whereas the factors that induce a more oxidative state in the prostate remain unclear.

The effect of experimental periodontitis on prostatic hyperplasia has not yet been reported. Under the framework of the hypothetical oral-prostate axis, the present study hypothesized that enhanced oxidative damage and inflammation might contribute to the aggravated pathogenesis of BPH by periodontitis. Herein, we established a rat model of ligature-induced periodontitis and testosterone-induced BPH to explore the possible relationship between periodontitis and BPH development. We also conducted an *in vitro* experiment on *Porphyromonas gingivalis* lipopolysaccharide- (LPS-) induced BPH-1 cells to determine whether oxidative stress is involved in this relationship, thus providing certain experimental evidence for the association of periodontitis with BPH and clarifying the potential mechanism linking the two diseases.

2. Materials and Methods

2.1. Animals. Male adult Sprague-Dawley (SD) rats weighting 300-350 g were obtained from the Animal Experiment Center of Zhongnan Hospital of Wuhan University and acclimated (temperature-controlled room on a half-day light/dark cycle with free access to water and food) for 1 week. All procedures in handling animals were adhered to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Wuhan University (IACUC animal approval protocol #2018119).

2.2. Experimental Design. The rats were randomly divided into four groups ($n = 5$/group): (1) a sham-operated group (control) undergoing sham operation for castration and subcutaneous injection of 0.9% saline solution (5 mg/kg/day) for four weeks, (2) a testosterone-induced BPH group (BPH) undergoing castration and subcutaneous injection of testosterone propionate (5 mg/kg/day) (Ningbo Second Hormone Factory, Ningbo, China) for four weeks [21], (3) a ligature-induced experimental periodontitis group (EP) undergoing ligation of sterile nylon thread around the cervical of bilateral maxillary first and second molars [22] and subcutaneous injection of 0.9% saline solution (5 mg/kg/day) for four weeks, and (4) a composite group (EP+BPH) undergoing castration and subcutaneous injection of testosterone propionate as the testosterone-induced BPH group; simultaneously, the maxillary first and second molars of rats were treated the same way as those of the ligature-induced EP group. The above surgical procedures were conducted under anesthesia by intraperitoneal injection of sodium pentobarbital (40 mg/kg), and all rats received subcutaneous injections in a week after the surgeries.

2.3. Harvest of the Rat Prostate. After 4 weeks of the injection administration, all rats were euthanized using an overdose of anesthetic. Prostatic ventral lobes and seminal vesicles were harvested, and the wet weight of the isolated prostate was measured immediately. Portions of the ventral prostate were kept in 4% paraformaldehyde (Dalian Meilun Biotechnology Co., Ltd., Dalian, China) for histological and immunohistochemical analyses. The remaining prostate tissues were frozen in liquid nitrogen for subsequent molecular analyses.

2.4. Microcomputed Tomographic (Micro-CT) Analyses of Alveolar Bone. Bilateral maxillary alveolar bone-contained molars were harvested and fixed with 4% paraformaldehyde. The right maxillae were dissected free of soft tissues to evaluate alveolar bone loss in each group by micro-CT (Skyscan 1176, Bruker, Kontich, Belgium). The X-ray generator was operated at a source voltage of 58 kV and source current of 431 μA with an exposure time of 1000 ms and an image pixel size of 9 μm. Three-dimensional (3D) reconstruction was performed by the software NReCon (Bruker, Kontich, Belgium) after scanning. 3D images of alveolar bones were obtained by the software CTvox (Bruker, Kontich, Belgium). The cementoenamel junction to alveolar bone crest (CEJ-ABC) was measured by DataViewer 1.5.2.4 (Bruker, Kontich, Belgium) for the maxillary second molar of four sites as follows: the proximal buccal, proximal palatal, distal buccal, and distal palatal. The CEJ-ABC distance was then calculated for statistical analysis. The residual left side of the maxillae was subsequently used for histological evaluation.

2.5. Histopathological Analyses. The fixed prostate tissues and periodontal tissues were embedded into paraffin after being dehydrated in gradient alcohol for dehydration. And then, 4 μm sections were prepared with a microtome (Leica Biosystems, Shanghai, China) for histological examinations. The H&E staining was conducted according to standard procedures, and images were obtained with a light microscope (Leica DFC295, Wetzlar, Germany).

Masson's trichrome staining was applied for the obtained prostate paraffin sections (4 μm) to highlight the microstructures of prostatic epithelia, smooth muscle (SM)

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cells, and collagen fibers (CF) after being stained red, dark red, and blue, respectively. Histological alterations of prostate tissues were observed by inverted phase contrast microscope (Cat. #DMi1, Leica, Wetzlar, Germany). The epithelial thickness of ventral prostate tissues and area percentages of epithelia, SM, and CF were quantified then analyzed by ImageJ software (ImageJ, National Institutes of Health, USA).

2.6. Immunohistochemistry Staining for Ki67. Tissue sections (4 μm) were prepared for immunohistochemical study. The sections were processed with deparaffinization, retrieval in EDTA solution (Aspen, Wuhan, China), and incubation in 3% hydrogen peroxide (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The samples were incubated overnight at 4°C with primary antibody of rabbit polyclonal anti-Ki67(Affinity Biosciences, Cincinnati, USA), then incubated with the HRP-labeled goat anti-rabbit second antibody (Aspen, Wuhan, China) at 37°C for 50 min. Brown-stained nuclei in cells were considered positive and the Ki67-positive rate was quantified by ImageJ software.

2.7. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay. The determination of prostate cell apoptosis was evaluated by terminal deoxynucleotidyl transferase- (TdT-) mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay according to the manufacturer's protocol. The deparaffinized prostate sections (4 μm) were digested with proteinase K (Roche molecular biochemical kit, Germany) and incubated in permeabilization buffer (Aspen, Wuhan, China). The tissues were further processed with the TUNEL reaction mixture (TdT and dUTP) and DAPI and finally were sealed by antifade fluorescence mounting medium (Aspen, Wuhan, China). The images of immunofluorescence staining were obtained with tissue cell nuclei being stained blue and TUNEL-positive nuclei stained green. Positive staining area and cell numbers were analyzed by ImageJ software, and the apoptosis rate (%) was estimated as the (number of positive cells/the total number of epithelial cells) $\times$ 100.

2.8. Western Blot Analysis for Bax and Bcl-2 Protein Expression. Prostatic tissues were homogenized using RIPA protein lysis buffer (Beyotime Biotechnology, Shanghai, China) in an ice bath. The supernatant of protein solution was collected after centrifugation (13,300 rpm for 30 min) at 4°C. Bicinchoninic acid (BCA) protein assay kit (Biosharp, Shanghai, China) was used to determine the protein concentrations of the samples. Western blot was performed with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein samples were separated by SDS-PAGE then transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked in 2% BSA for 1 h then incubated with the rabbit monoclonal anti-Bax primary antibody at 1 : 1000 (Abcam, Cambridge, USA) or rabbit polyclonal anti-Bcl-2 primary antibody at 1 : 1000 (Abcam, Cambridge, USA) at 4°C overnight. Then, membranes were incubated with HRP-conjugated goat anti-rabbit second antibody at 1 : 5000 (Boster, Wuhan, China) at room temperature for 1 h after washing. GAPDH (Abcam, Cambridge, USA) was used as a loading control. Thereafter, immunodetection bands were reacted with an Enhanced Chemiluminescence (ECL) kit (Beyotime Biotechnology, Shanghai, China) and analyzed with an Alliance Q9 Chemiluminescence Imager (Uvitec, UK). The list of antibodies used for western blot analysis is presented in Table 1.

2.9. Determination of Inflammatory Cytokines. Blood samples were collected from an abdominal aorta before the rats were sacrificed, and plasma was separated by centrifugation (3000 rpm for 15 min) at 4°C. Plasma samples were analyzed for tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and C-reaction protein (CRP) using ELISA kits (ELK Biotechnology, Wuhan, China) according to the manufacturer's instructions.

2.10. Determination of Oxidative Stress Markers

2.10.1. Human Prostatic Cell Line. Human benign prostatic enlargement epithelial cell line BPH-1 was obtained from the Department of Urology in Zhongnan Hospital of Wuhan University and cultured as previously described [23].

2.10.2. Determination of ROS. The production of ROS induced by *Porphyromonas gingivalis* LPS was evaluated following the ROS assay kit (Dojindo, Kumamoto, Japan) manual. Briefly, 1×10^5 BPH-1 cells/well were seeded into 6-well plates with RPMI-1640 (Hyclone, USA), containing 10% fetal bovine serum (Hyclone, USA), and cultured at 37°C for 24 h. The medium was replaced with RPMI-1640 containing 1 μg/mL *Porphyromonas gingivalis* LPS (Sigma, SMB00610) and cultured at 37°C for a further 24 h. The supernatant was removed, and highly sensitive DCFH-DA dye working solution (Dojindo, Kumamoto, Japan) was added then incubated at 37°C for 30 min. The changes in the levels of ROS were detected using flow cytometry.

2.10.3. Determination of H_2O_2. The concentration of H_2O_2 induced by *Porphyromonas gingivalis* LPS was evaluated using the H_2O_2 assay kit (Beyotime Biotechnology, Shanghai, China). BPH-1 cells were treated with 1 μg/mL *Porphyromonas gingivalis* LPS for 24 h as above and collected into a centrifuge tube, then H_2O_2 lysis was added for homogenization of cells. The supernatant was collected after centrifuging at 12000 g for 5 min at 4°C. Then, a 50 μL sample or standards and a 100 μL hydrogen peroxide detection reagent were added and incubated at room temperature for 30 min. The changes in the levels of H_2O_2 were detected using a microplate reader (Thermo Scientific Varioskan Flash).

2.11. Statistical Analysis. The data were expressed as the means ± standard error of mean (SEM). Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The differences between groups were analyzed with an independent-sample test or one-way analysis of variance (ANOVA); Bonferroni correction was applied for post hoc comparisons. $p < 0.05$ was considered statistically significant.

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Table 1: List of antibodies used for western blot analysis.

Primary antibodies	Source species	Antibody supplier	Dilution buffer	Dilution ratio	Dilution buffer supplier
GAPDH	Rabbit	Abcam, ab181602	QuickBlock™ Primary Antibody Dilution Buffer	1 : 1000	Beyotime, P0256
Bax	Rabbit	Abcam, ab182733	QuickBlock™ Primary Antibody Dilution Buffer	1 : 1000	Beyotime, P0256
Bcl-2	Rabbit	Abcam, ab196495	QuickBlock™ Primary Antibody Dilution Buffer	1 : 1000	Beyotime, P0256
Secondary antibody					
HRP-goat anti-rabbit		Boster, BA1054	1 × TBST	1 : 5000	Solarbio, T1081

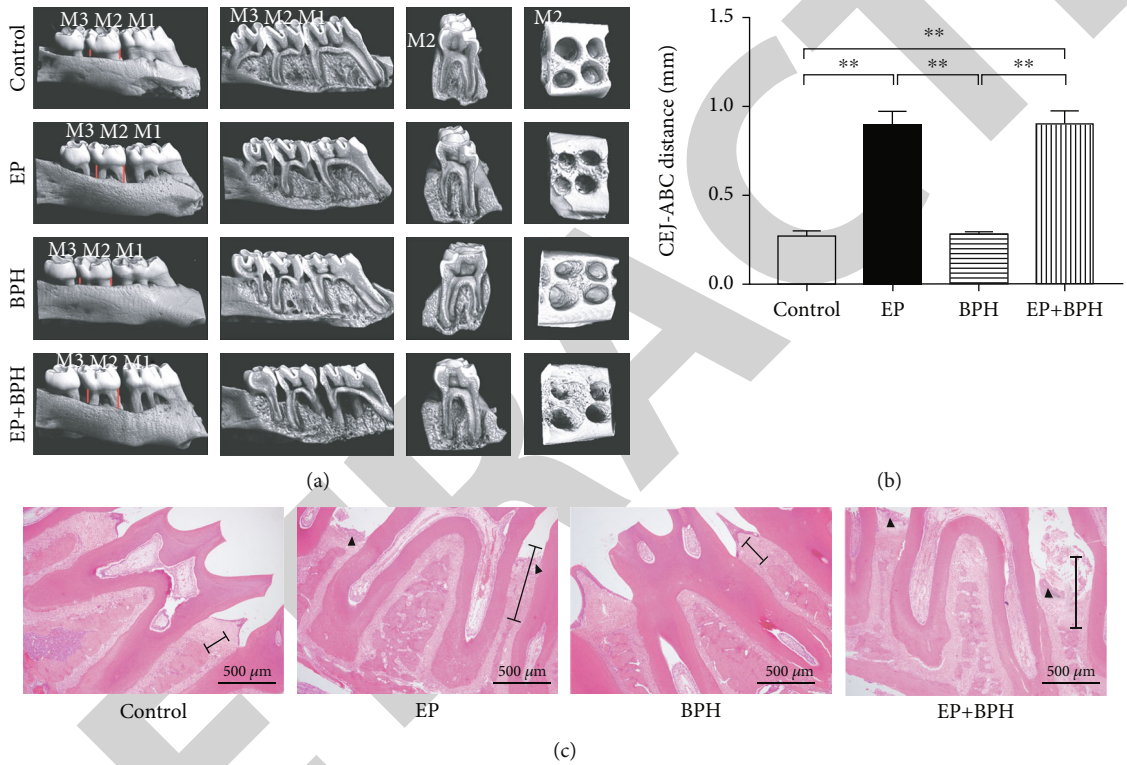

Figure 1: Alveolar bone loss and histological changes in rat periodontal tissues. (a) Representative images obtained by micro-CT, including the surface of the bone, the trabecular structure inside, the regain of root furcation of the second molar, and the bone surrounding the roots. The red lines showed the linear distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) for the maxillary second molar. (b) Quantitative analysis of the CEJ-ABC distance from the control, EP, BPH, and EP+BPH groups. Data are presented as mean ± SEM (**$p < 0.01$). (c) Representative figures from HE staining for the second maxillary alveolar bones (arrows indicate inflammatory changes in the gingival epithelium, scale plates indicate linear distance from CEJ to ABC). Scale bar = 500 μm; original magnification ×40.

3. Results

3.1. Micro-CT and Histopathological Analyses of Alveolar Bone. Experimental periodontitis was established in the EP model and EP+BPH model, which was confirmed by the analyses of micro-CT. The 3D images provided the clear morphology views of the maxillary alveolar bones from different angles (Figure 1(a)), which showed obvious bone resorption, especially for the residual ridge between the first molar and the second molar. Statistical analysis also demonstrated that the CEJ-ABC of the animals in EP and EP+BPH was significantly increased when compared with the control and BPH groups (control vs. EP/EP+BPH: $p < 0.01$; BPH vs. EP/EP+BPH: $p < 0.01$) (Figure 1(b)). Our results indicated that all rats from the EP and EP+BPH groups were successfully induced to the ligature-induced experimental periodontitis. Periodontal histopathologic analysis of the regions of the second molar showed the structures of gingiva, periodontal ligament, alveolar bone, and cementum. The rats suffered experimental periodontitis (EP and EP +BPH groups) and observed the erosion of gingival epithelium with infiltrated inflammatory cell, derangement of collagen fiber within the periodontal ligament, loss of epithelial attachment, and breakdown of alveolar bone (Figure 1(c)).

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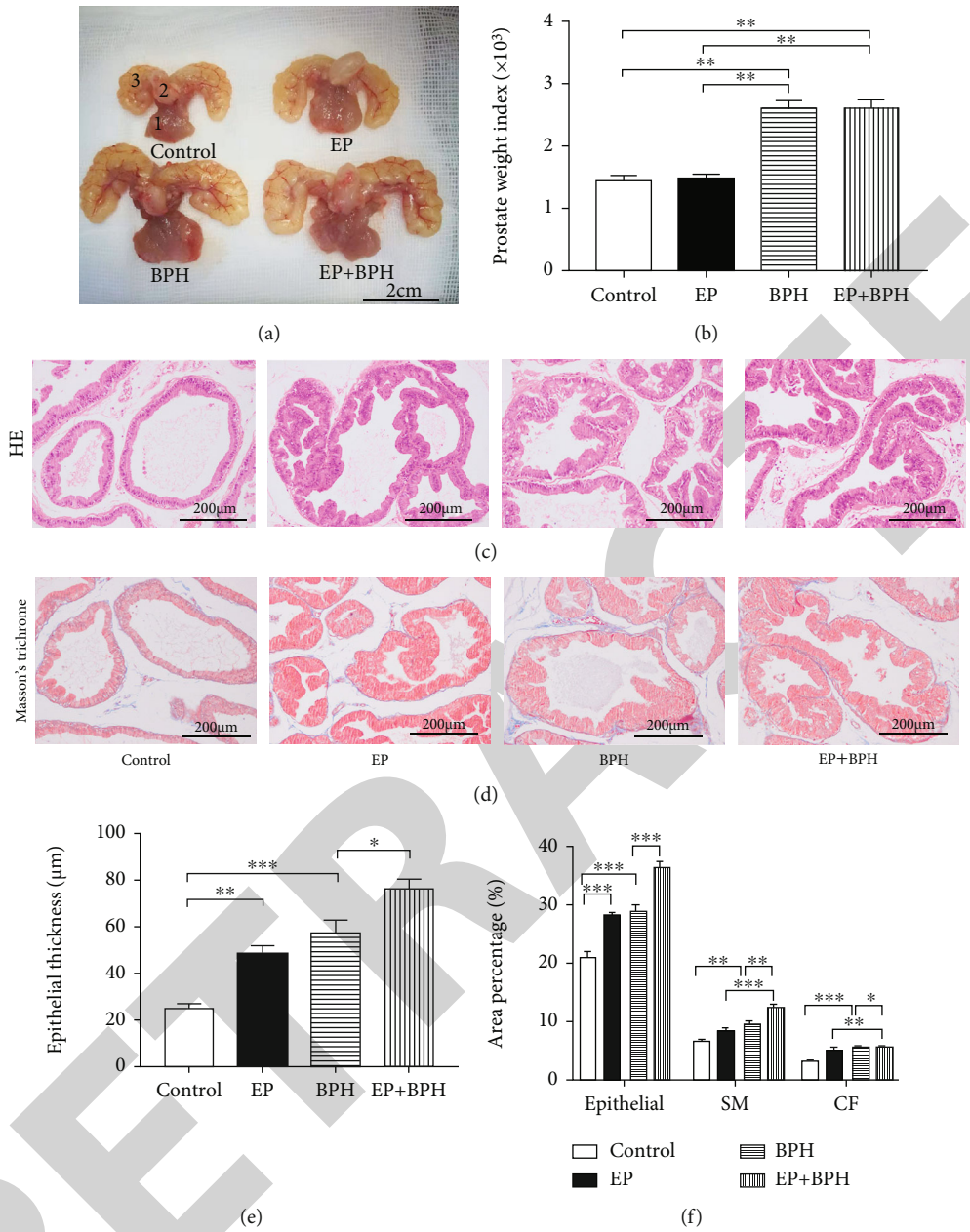

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Figure 2: Typical photographs and histological examination of rat prostate tissues. (a) Photographs of (1) ventral prostate, (2) bladder, and (3) seminal vesicle from the control, EP, BPH, and EP+BPH groups. (b) A bar graph for prostate weight index (prostate weight of each animal/body weight of each animal) × 1000. (c) Representative figures from H&E staining for the prostate tissues. (d) Representative figures from Masson's trichrome staining for the prostate tissues. Prostatic epithelial cells, smooth muscle (SM) cells, and collagen fibers (CF) were stained red, dark red, and blue, respectively. Scale bar = 200 μm; original magnification ×200. (e) A bar graph for epithelial thickness of ventral prostate tissues. (f) A bar graph for area percentage of different components (epithelia, SM, and CF) from the control, EP, BPH, and EP+BPH groups. Data are presented as mean ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

And no obvious pathological change was found between the BPH group and the control group.

3.2. Effects of Experimental Periodontitis on the Histological Alteration of Prostate Tissues

3.2.1. Prostate Weight and Index. All the rats undergoing castration and induced testosterone showed a significant increase in prostate volume. Prostate enlargement in rats can be induced in testosterone-treated rats, which was aggravated by periodontal ligation (Figure 2(a)). Prostate weight and index were significantly enhanced by 66% and 80% in testosterone treated rats, respectively, when compared to the control group. Simultaneous periodontitis caused an obvious increase in the prostate weight by 59% and the prostate index by 81%, respectively, when compared to the control group (Figure 2(b)). Body weight was measured before the experiments and after the 4-week injection. At the end

Table 2: Variation of physiological parameters in rat models.

Group	Body weight (g)		Prostate weight (mg)	Prostate weight index ($\times 10^3$)
	Initial	Final		
Control	304.60 ± 5.99	424.60 ± 12.36	609.00 ± 23.18	1.44 ± 0.08
EP	298.20 ± 4.22	412.20 ± 9.09	613.20 ± 32.16	1.49 ± 0.06
BPH	306.00 ± 15.21	393.80 ± 21.04	1009.4 ± 26.10***	2.59 ± 0.14***
EP+BPH	298.00 ± 8.44	370.40 ± 5.22	969.0 ± 61.01***	2.61 ± 0.13***

Prostate weight index = (prostate weight of each animal/body weight of each animal) × 1000. Data are presented as mean ± SEM. ***$p < 0.001$ vs. control rats; ***$p < 0.001$ vs. EP rats.

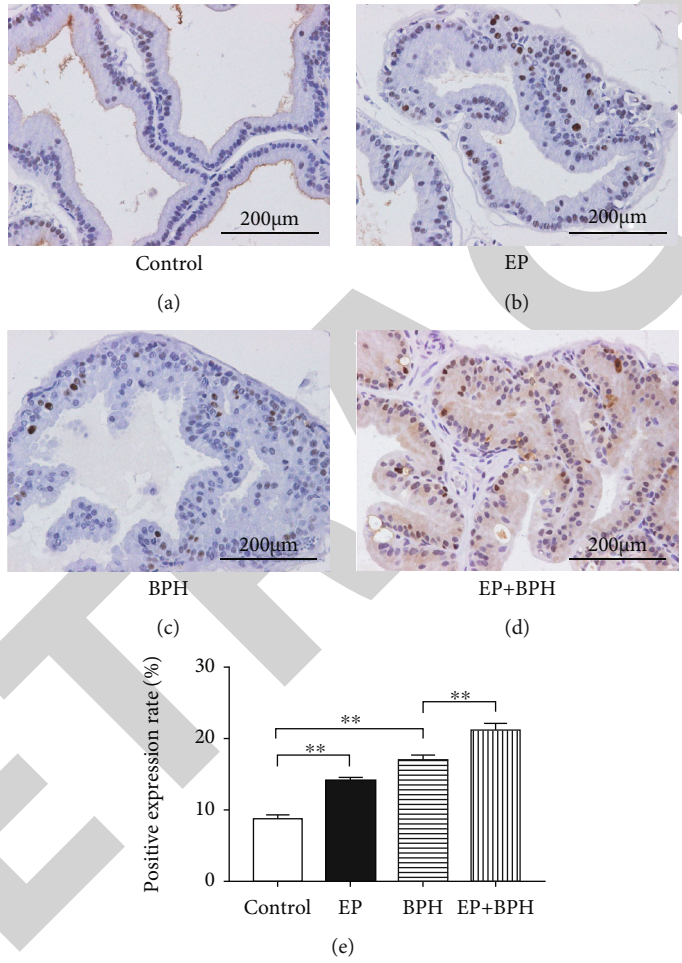

Figure 3: Ki67 staining for detecting cell proliferation of prostate tissues. (a–d) Representative figures from Ki67 immunohistochemical staining for ventral prostates of the control, EP, BPH, and EP+BPH groups, respectively. Scale bar = 200 μm; original magnification ×400. (e) A bar graph for rate (%) of Ki67-positive cells in rat prostate. Data are presented as mean ± SEM (**$p < 0.01$).

of the experiment, rats in the composite group showed the lowest body weight, followed by those in the BPH and EP groups (Table 2).

3.2.2. Histopathological Examination of Ventral Prostate Tissues. Microscopic examination (H&E staining) showed that prostatic tissues obtained from control rats remain as a normal structure with numerous acini containing homogenous acidophilic material (Figure 2(c)), whereas prostates taken from rats injected with testosterone displayed marked glandular hyperplasia and a decreased glandular luminal area compared to the control animals (Figure 2(c)). Masson's trichrome staining showed that testosterone-treated rats displayed increased stromal cells in the prostate (Figure 2(d)). Quantitative analysis of histological components suggested that the EP group presented with a significantly thickened epithelium; the BPH group showed increased epithelium, SM, and CF; and the EP+BPH group demonstrated the most obvious increasing of hyperplasia of epithelia, SM, and CF, with all $p < 0.05$ (Figures 2(e) and 2(f)).

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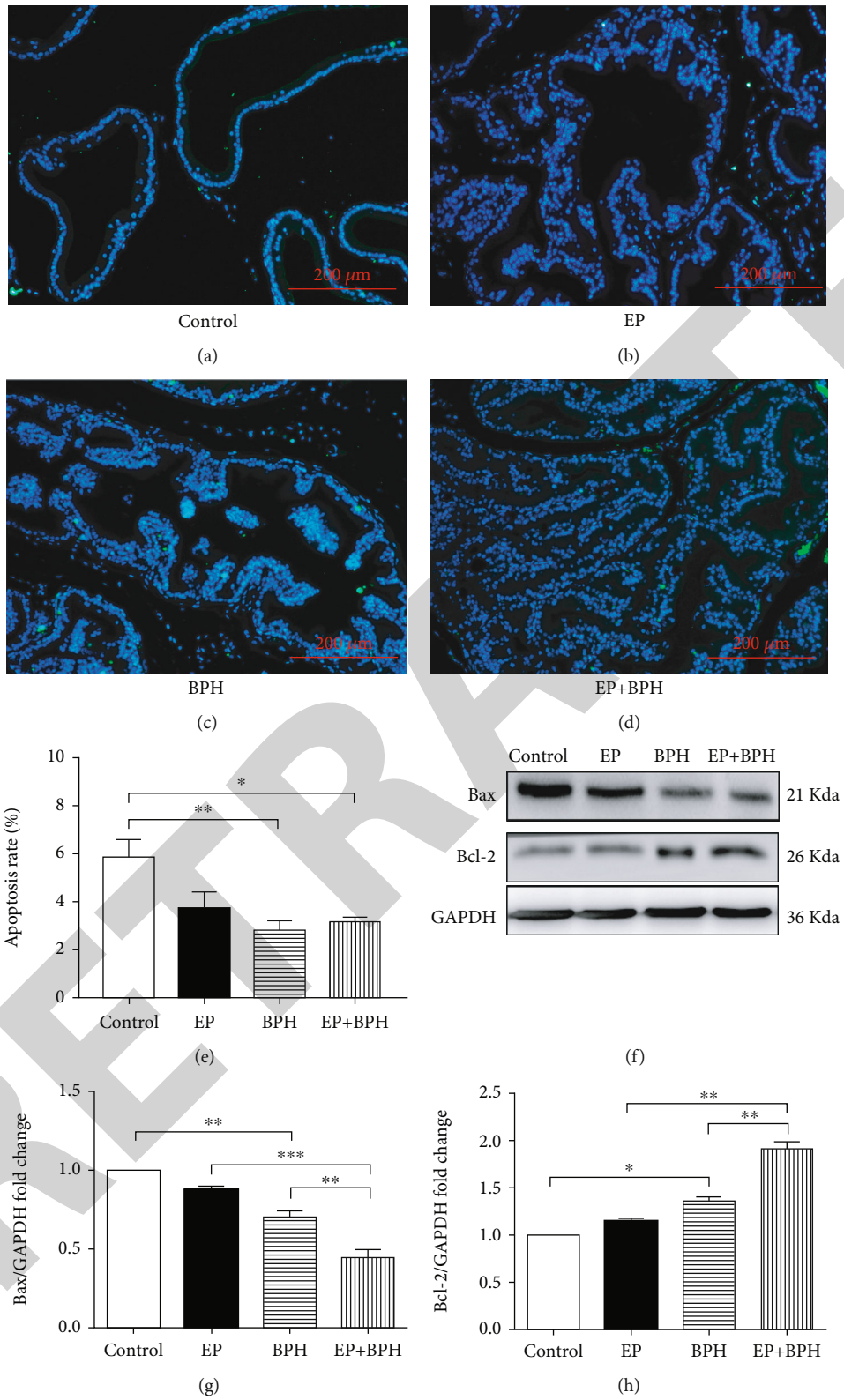

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Figure 4: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and western blot analysis for detecting cell apoptosis of prostate tissues. (a–d) Representative figures from TUNEL staining of prostate tissues for the control, EP, BPH, and EP+BPH groups, respectively. Tissue cell nuclei were stained blue with DAPI; TUNEL-positive nuclei were stained green. Scale bar = 200 μm; original magnification ×200. (e) A bar graph for apoptosis rate (%) of TUNEL-positive cells in rat prostate. (f) Representative protein bands for Bax and Bcl-2 in prostate tissues among the indicated groups. (g) Quantitative analysis of the protein level of Bax relative to GAPDH. (h) Quantitative analysis of the protein level of Bcl-2 relative to GAPDH. Data are presented as mean ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

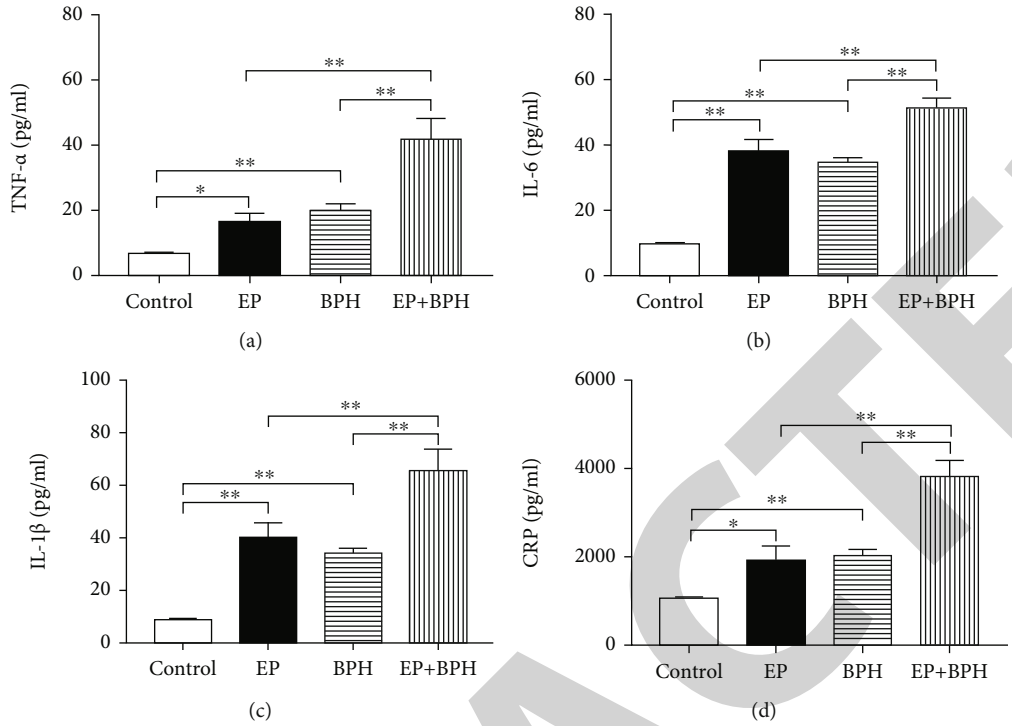

Figure 5: Enzyme-linked immunosorbent assay (ELISA) for detecting the inflammatory cytokine levels of (a) TNF-α, (b) IL-6, (c) IL-1β, and (d) CRP in plasma of rats. TNF-α: tumor necrosis factor-α; IL: interleukin; CRP: C-reaction protein. Data are presented as mean ± SEM ($^*p < 0.05$, and $^{**}p < 0.01$).

3.3. Effects of Experimental Periodontitis on the Proliferation and Apoptosis of Prostate Tissues

3.3.1. Immunohistochemical Detection of Ki67. The proliferation marker Ki67 protein expression was evaluated immunohistochemically and showed 8.80% positive rate in the control group (Figure 3(a)). However, in the group which received periodontal ligation or testosterone alone, an increment of stained cells was noted (approximately 14.01% and 17.08%), suggesting an elevation of the proliferation rate (Figures 3(b) and 3(c)). Simultaneous periodontitis and BPH further increased the number of Ki67-positive cells (Figure 3(d)), in comparison to the BPH rats. The quantitative analysis results (Figure 3(e)) indicated that the Ki67-positive rate was significantly higher in the experimental groups than in the control, among which the EP+BPH group was at the highest degree of proliferation. In addition, significant differences were detected between the groups (all $p < 0.01$).

3.3.2. Determination of Apoptosis of Prostate Tissue. TUNEL assay showed obvious characteristic of antiapoptosis of prostate cells in the testosterone-treated rats (Figures 4(a)–4(d)). The quantitative analysis also revealed a significant decrease of the apoptosis rate in BPH and EP+BPH groups (Figure 4(e)). The protein expression levels of Bax and Bcl-2 were also detected to investigate the cell apoptosis of the rat prostate (Figure 4(f)). The apoptosis inducer Bax was shown to be downregulated and the apoptosis inhibitor Bcl-2 upregulated in the experimental groups compared with the control (Figures 4(g) and 4(h)), which was obviously observed in the composite group, which further confirmed the explained antiapoptosis of prostate cells in testosterone-treated rats.

3.4. Effects of Experimental Periodontitis on Plasma Levels of Inflammation Cytokines in BPH Rats. Periodontitis and BPH both significantly enhanced inflammatory marker levels of TNF-α, IL-6, IL-1β, and CRP in plasma when compared to the control group. Simultaneous periodontitis and BPH synergistically further enhanced inflammation cytokine levels than when treated alone (Figure 5), which indicate a potential effect of experimental periodontitis on systemic inflammation levels in BPH rats.

3.5. Effects of Porphyromonas gingivalis LPS on the Generation of ROS and H_2O_2 in BPH-1 Cells. Our results showed that *Porphyromonas gingivalis* LPS increased the intracellular levels of ROS in BPH-1 cells after 24 h of exposure (Figures 6(a) and 6(b)), with significant difference from the untreated group (Figure 6(c)). In line with the enhanced generation of ROS, the concentration of H_2O_2 also significantly increased after *Porphyromonas gingivalis* LPS exposure for 24 h (Figure 6(d)).

4. Discussion

The high prevalence of BPH in patients with periodontitis has become a major worldwide health problem affecting elderly male populations. Thus, it is quite crucial to clarify

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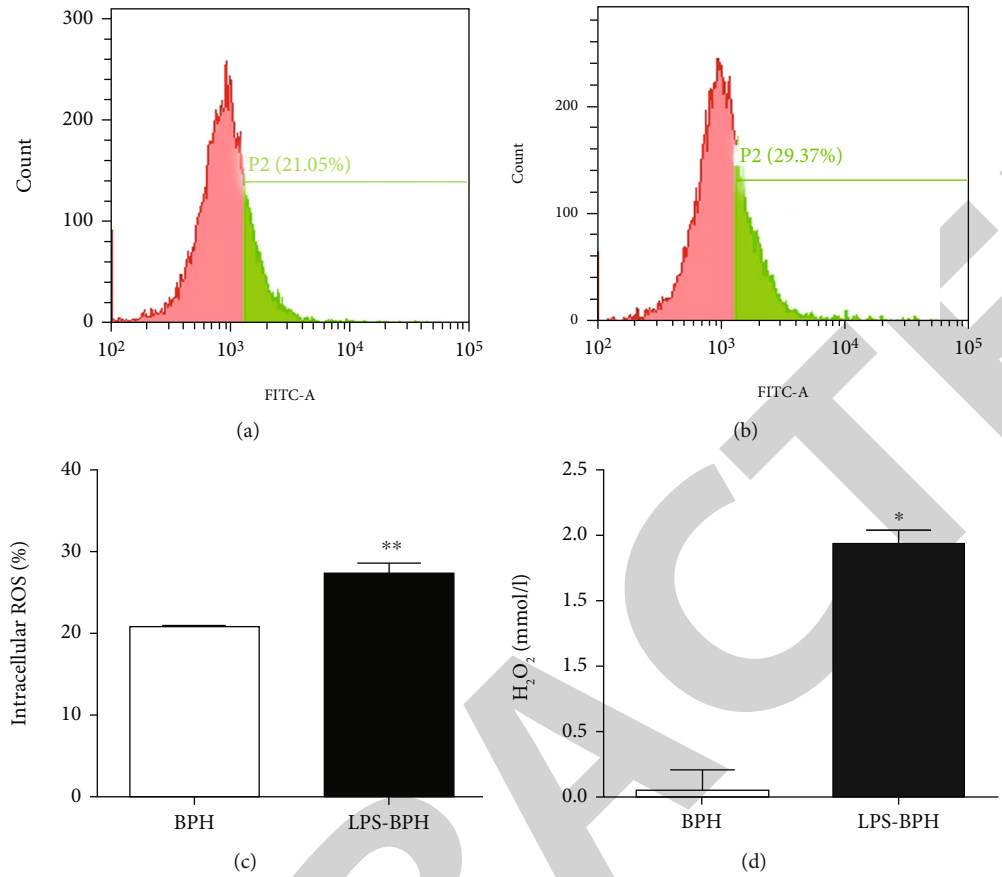

Figure 6: Assessment of intracellular ROS and H_2O_2 production in BPH-1 cells treated with *Porphyromonas gingivalis* LPS. The effect of *Porphyromonas gingivalis* LPS on the production of ROS was evaluated by flow cytometry in BPH-1 cells, either (a) untreated control or (b) *Porphyromonas gingivalis* LPS exposure for 24 h. (c) A bar graph for intracellular level of ROS (%) in BPH-1 cells, either untreated (BPH) or treated with *Porphyromonas gingivalis* LPS for 24 h (LPS-BPH). (d) A bar graph for intracellular expression of H_2O_2 in BPH-1 cells, either untreated (BPH) or treated with *Porphyromonas gingivalis* LPS for 24 h (LPS-BPH). ROS: reactive oxygen species; H_2O_2: hydrogen peroxide. Data are presented as mean ± SEM ($^*p < 0.05$, and $^{**}p < 0.01$).

the mechanism accounting for the aggravation of BPH by periodontitis. In this study, for the first time, we established rat models to examine the effect of experimental periodontitis on prostatic hyperplasia, which provide the evidence that periodontitis may promote the development of BPH through the regulation of oxidative stress and systemic inflammation.

In previous animal studies, it was proven that injection of testosterone is an established model for induction of BPH in rats [21], and periodontal ligation is an established model for induction of periodontitis in rats [22]. To our knowledge, this is the first study examining the potential role of periodontitis in the development of prostatic hyperplasia based on animal models. In our study, comparison models were successfully constructed and verified by histopathological findings. Testosterone-induced hyperplasia indicated a significant increase in prostate weight and prostate index. Periodontitis significantly promoted testosterone-induced hyperplasia which was indicated by the aggravation of the pathological structure alterations in the composite model. Histologically, the observation of pathology changes in testosterone-treated groups, especially in EP+BPH, indicated a predisposition to BPH and an aggravated effect of periodontitis on prostate hyperplasia. The imbalance of cell proliferation and apoptosis is known to be involved in the development of BPH [24]. Furthermore, the Bax/Bcl-2 ratio has been widely studied as an influential factor for progression of apoptosis in developing BPH [25]. This is consistent with our results, as experimental groups present increased Ki67 expression and decreased apoptosis of prostate cells. Additionally, our study found a downregulated Bax and upregulated Bcl-2 expression in the experimental groups, in which the most obvious changes were detected in the EP+BPH group, which is consistent with the manifestations of BPH [26, 27] and further defined the effect of periodontitis on the apoptosis of prostate tissues.

Accumulated research has confirmed the inflammatory association between periodontitis and BPH based on epidemiological investigation or clinical data, which probably occurs through dissemination of proinflammatory cytokines that can further exaggerate the preexisting inflammation [28, 29]. Experimentally, numerous researches have confirmed inflammation as one of the factors by which testosterone can induce BPH [30, 31]. In this study, the markedly raised inflammatory cytokines of IL-6, IL-1β, TNF-α, and CRP in testosterone-induced models could support the hypothesis.

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Also, the role of oxidative stress was highlighted in testosterone-induced BPH [32]. Testosterone has been reported to increase prostatic cellular metabolism, thus inducing a wide production of free radicals in combination with dampening the antioxidant system, leading to cell dysfunction [33]. Our data found that LPS from *Porphyromonas gingivalis* induced a significantly higher level of intracellular ROS and H_2O_2 in BPH-1 cells, indicating oxidative stress might provide a molecular link between periodontal disease and BPH development.

Oxidative stress and inflammation underpin several age-related diseases, and their mechanisms are inextricably linked. For instance, oxidative stress may increase the production of proinflammatory cytokines thus triggering systematic inflammation [34]. Anti-inflammatory cascades are linked to decreased oxidation, both playing key roles in cellular and tissue damage, influencing the development of multiple age-related diseases [35, 36]. Inflammation and oxidative stress significantly contribute to prostatic remodeling. Although the factors that produce a more oxidative state in the prostate remain unclear, studies indicated that the hormonal environment (i.e., testosterone) and chronic inflammation might be relevant in this process [37]. Indeed, the onset of prostatic tissue inflammation and oxidative stress imbalance can result in the accumulation of inflammatory cytokines and other growth factors [38]. This is in line with our noticed increase in the plasma levels of inflammatory cytokines in testosterone-treated rats. Furthermore, it is consistent with their famous role in promoting prostate cell proliferation.

5. Conclusions

In conclusion, our findings demonstrate that periodontitis might promote BPH development through regulation of oxidative stress and inflammatory process. The present study brings early evidence to this field; however, more studies should be done to clarify the role of these specific bacteria and their potential to be exploited as new biomarkers for BPH. This may help to reveal new strategies to combat BPH.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interests.

Authors' Contributions

Cheng Fang and Lan Wu contributed equally to this work.

Acknowledgments

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Retraction

Retracted: Mesenchymal Stem Cell-Derived Exosomes and Their Potential Agents in Hematological Diseases

Oxidative Medicine and Cellular Longevity

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope
(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
(4) Inappropriate citations
(5) Incoherent, meaningless and/or irrelevant content included in the article
(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] M. Shen and T. Chen, "Mesenchymal Stem Cell-Derived Exosomes and Their Potential Agents in Hematological Diseases," *Oxidative Medicine and Cellular Longevity*, vol. 2021, Article ID 4539453, 13 pages, 2021.

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Review Article

Mesenchymal Stem Cell-Derived Exosomes and Their Potential Agents in Hematological Diseases

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Mesenchymal stem cells (MSCs) are the most exploited stem cells with multilineage differentiation potential and immunomodulatory properties. Numerous lines of findings have reported their successful applications in a multitude of inflammatory conditions and immune disorders. However, it is currently discovered that these effects are mainly mediated in a paracrine manner by MSC-exosomes. Moreover, MSC-exosomes have been implicated in a wide variety of biological responses including immunomodulation, oxidative stress, tumor progression, and tissue regeneration. Meanwhile, they are reported to actively participate in various hematological diseases by the means of transferring different types of exosomal components to the target cells. Therefore, in this review, we briefly discuss the sources and biological features of MSCs and then illustrate the biogenesis and biological processes of MSC-exosomes. Of note, this paper especially highlights the latest research progress of MSC-exosomes in hematological diseases.

1. Introduction

Mesenchymal stem cells (MSCs) are recently the most extensively studied stem cells [1–3], and MSC-based products are undergoing a rapid expansion [4–6]. It uncovered more than 1000 clinical trials when we searched the keywords "mesenchymal stem cell" or "mesenchymal stromal cell" in the ClinicalTrials.gov database (http://www.clinicaltrials.gov/, accessed on June 2021). Despite the tremendous achievements made in MSCs therapy [3, 7–11], there are several limitations toward their clinical translation, such as invasive cell collection procedures, orchestrated engraftment steps, low posttransplantation cell viability, poor homing, and multiple doses to maintain the therapeutic effects [12–15]. Promisingly, accumulating experimental and clinical studies reveal that the powerful therapeutic agents of MSCs are mainly exerted by their paracrine effects, in particularly by exosomes [13, 16–19].

Exosomes are 30 ~ 150 nm extracellular vesicles (EVs) and membrane bound nanoparticles that contain bioactive substances like proteins, DNA, RNAs, and cytokines [20–22]. These bioactive components play key roles in intercellular, intertissue, and cross-species communications [20] and participate in formation and progression of tumor microenvironment (TME) remodeling, intracellular homeostasis, and drug resistance [23, 24]. Nowadays, explosive evidence has shown that exosomes derived from MSCs exert biological effects on a variety of diseases, including models of myocardial infarction [25], hepatic fibrosis [26], inflammatory diseases [14], graft versus host disease (GVHD) [27, 28], novel coronavirus disease (COVID-19) [7, 13, 29], and hematological tumors [30–33]. As part of hematopoietic niche, the MSC-exosomes construct a biological microenvironment that maintains the homeostasis and responds to the oxidative stress, damage, and disease conditions [34–36].

MSC-exosomes, particularly when the homeostasis is disrupted, can carry complex cargoes and regulate homeostasis within disorders or cancers. In recent years, the administration of MSC-exosomes has yielded profound effects in a variety of hematological diseases including GVHD [37], multiple myeloma (MM) [38], acute myeloid leukemia (AML) [39], chronic myeloid leukemia (CML) [32], chronic lymphocytic leukemia (CLL) [40], lymphoma [31], and myelodysplastic syndrome (MDS) [41]. However,

these effects on target cells are two-edged sword. In some cases, MSC-exosomes can inhibit the tumor growth and disease progression [42, 43], while in other circumstances, they exert their cell-protecting and tumor-promoting effects [38, 44]. These controversial results of MSC-exosomes have been widely discussed in hematological diseases though without unifying conclusion.

Considering the diversified modes of action and the demands of precision medicine, we aim to put forward an overview of current knowledge of MSC-exosomes, to clarify their complex interactions with microenvironment niche, and to illuminate the therapeutic agents of MSC-exosomes in human hematological diseases.

2. General Characteristics and Functions of MSCs

2.1. Biological Sources of MSCs. MSCs are multipotent non-hematopoietic stem cells and have been intensely investigated for clinical applications within the last decades [16, 45, 46]. Since their first discovery from bone marrow by the Russian haematologist Friedenstein in 1970s [47], MSCs have been isolated from numerous tissues and various organs like bone marrow (BM), umbilical cord, placenta, amniotic fluid, adipose, dental pulp, and induced pluripotent stem cells (iPSCs) or human embryonic stem cells (ESCs) (Figure 1(a)) [45, 48]. To better study this population, it is of paramount importance to identify the phenotypes and characteristics of primary MSCs. Since no single biomarker is specific for the precise identification, the International Society for Cellular Therapy established the minimum but widely accepted criteria in 2006 [46, 49]: (i) plastic-adherent stromal cells with self-renewal capability; (ii) immunophenotype strongly positive for a cluster of surface makers CD105, CD73, and CD90, while negative for CD45, CD34, CD14, CD11b, and CD19 (Figure 1(b)) and (iii) multipotency with osteogenic, chondrogenic, adipogenic, and myogenic differentiation potential (Figure 1(c)). Nevertheless, MSCs are far from being a uniform cell type and inherited heterogeneities exist in different subsets [45, 49], which makes these criteria insufficient to standardize MSCs [50].

2.2. MSC Biological Features. MSCs play multiple roles related with pathological and physiological process of cells. It is well known that they obtain powerful immunomodulatory properties and high regenerative capacities to restore cellular homeostasis and tissue damage by producing a vast number of bioactive substances including exosomes [51, 52]. Of course, the most attractive trait of MSCs is the immune compatibility between donors and recipients, which provides a safe haven for cell therapy and lowers risks of transferred cell rejection such as GVHD [53, 54]. Nevertheless, MSCs are actually not immune privileged [55], especially when exposed to inflammatory cues and oxidative stress settings in vivo, and MSCs in turn constitutively increase immunogenicity and further decrease viability and differentiation capacity [56]. In addition, during the processes MSCs cultured in vitro, MSC immunogenicity can be further triggered and amplified by inappropriate processes and culture conditions [45]. Factors contributing to ultimate failure of MSCs clinical translation may include but are not limited to the poor-quality, heterogeneity differentiation and immune compatibility [57]. Notwithstanding, these obstacles in turn promote a surge of interest towards MSC-exosomes and make them powerful candidates for cell-free therapy [27, 28, 58–60].

3. Constituents of Exosomes and Characteristics of MSC-Exosomes

3.1. Exosomes Biogenesis. Exosomes are secreted, membranous, metabolically active platforms, which are formed through the fusion of multivesicular bodies (MVBs) with the endocytic machinery (Figure 1(b)). Though parts of MVBs fuse with the lysosome for degradation [23], a subpopulation of MVBs fuse with the plasma membrane and release intraluminal vesicles as exosomes [61, 62]. Exosomes encapsulate various types of biomolecules including proteins, lipids, messenger RNA (mRNAs), DNA, microRNA (miRNAs), long noncoding RNA (lncRNA), and metabolites (Figure 1(d)) and are subsequently delivered into the extracellular space [63, 64]. These constitutive elements have been identified from different cell types and play different roles, which illustrate their compositional complexity and functional diversity. Afterwards, a surge increase of reviews has summarized their contents and corresponding functions [21, 23, 61, 65, 66], and various online databases (ExoCarta, http://www.exocarta.org/; Vesiclepedia, http://microvesicles.org/; exoRBase, http://www.exoRBase.org) have cataloged the proteins, lipids, and RNAs of exosomes.

3.2. Components of Exosomes. Exosomes are enriched in proteins with highly heterogeneous functions (Figure 1(d)). Tetraspanins (CD9, CD81, and CD63) take part in cellular interactions by binding with molecules integrins and MHC [67, 68]; heat shock proteins (HSP70, HSP90, HSP27) are involved in the process of antigen bind and presentation [69]; metabolic enzymes (fatty acid synthase, phosphoglycerate kinase, ATPase) maintain homeostasis [70]. Aside from functional proteins, more attentions are focused on exosomal RNAs. They are now the newest extracellular vesicle components to be discovered [65]. The abundant exosomal RNAs contain mRNA, circular RNA (circRNA), lncRNA, and miRNA [65]. Moreover, their contents differ both in quantity and in composition depending on the cellular types and microenvironmental niches and can be incorporated into recipient cells to function [67, 71]. Exosomes are also enriched in lipids such as sphingomyelin, lysophospholipids, gangliosides, and cholesterol [72]. Remarkably, these diverse lipids not only constitute a distinct structure for exosomal membranes but also are essential elements in exosomal biogenesis and release [73]. Altogether, the bioactive components in exosomes not only can be used as hallmark signatures but also can participate in various biological processes such as exosome biogenesis, metabolism, and antigen presentation.

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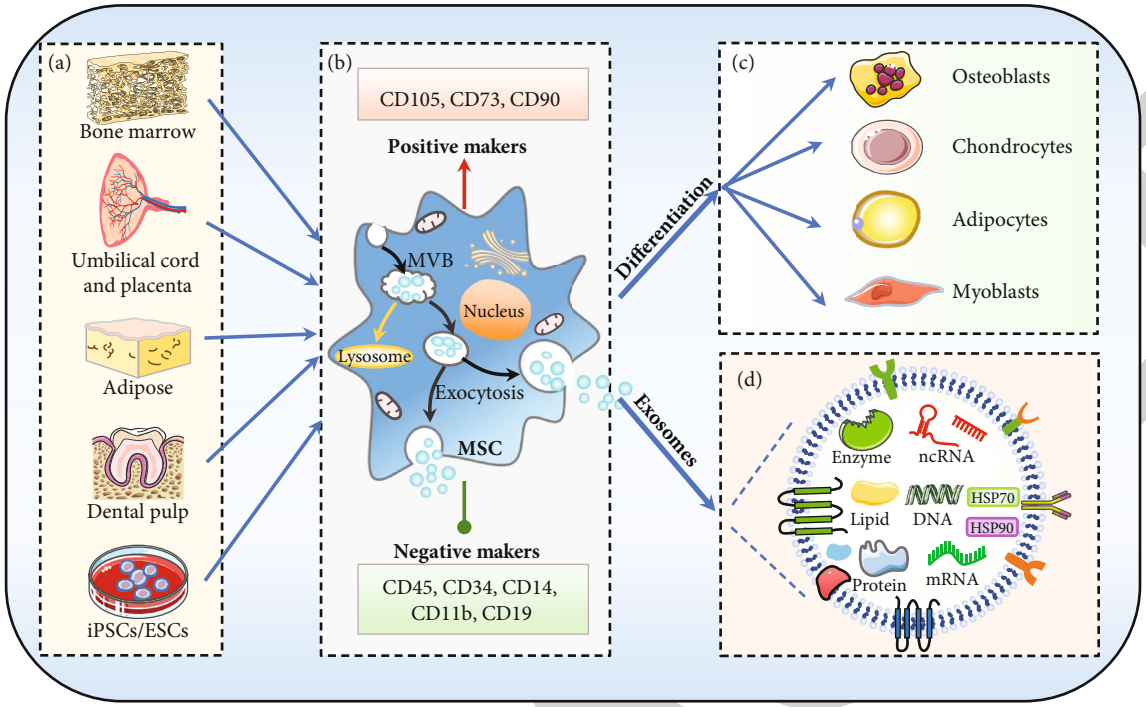

Figure 1: The biogenesis and release of MSC-exosomes. (a) The multiple sources of MSCs: MSCs can be isolated from the bone marrow, umbilical cord, placenta, adipose, dental pulp, and iPSCs/ESCs. (b) The molecular identification of MSCs and biogenesis of MSC-exosomes: MSCs are positive for the makers of CD105, CD73, and CD90 but negative for CD45, CD34, CD14, CD11b, and CD19. MSC-exosomes are formed through the fusion of MVBs and secreted into the extracellular space. (c) MSCs can differentiate into osteoblasts, chondrocytes, adipocytes, and myoblasts. (d) MSC-exosomes are enriched in multiple bioactive components including proteins, mRNAs, DNA, lipids, miRNAs, lncRNA, and metabolites. iPSCs/ESCs: induced pluripotent stem cells/embryonic stem cells; MVB: multivesicular body; mRNA: messenger RNA; ncRNA: noncoding RNA; HSP: heat shock proteins.

3.3. Characteristics of MSC-Exosomes. MSC-exosomes, with the same topology as their counterpart live cells [21], have attracted considerable attention [26, 29]. By taking advantage of nanotherapeutic agent, they have shown results similar to MSCs transplantation while avoiding risks related to cell-based therapy [74]. Owing to their acellular structure, they are in nature nontoxic and less immunogenic and have the abilities to shield from chemical and enzymatic degradations as well as to evade subsequent recognition and elimination by the immune system [20]. After being secreted or released into the extracellular milieu, they can easily permeate through various biological barriers at a higher rate such as blood-brain barrier (BBB) [23, 75]. Besides, these released exosomes from blood and other bodily fluids can be used as circulating biomarkers for cancers and diseases [61, 65]. As for precision medicine, the bioengineered exosomes can be relatively easier to manipulate and modify to target the specific cells [20, 76]. By virtue of their small size, low immunogenicity, long half-life, and ease with which they can be obtained, MSC-exosomes are thereby poised to become a rising star as effective delivery vehicles [46].

4. Biological Therapy Agents of MSC-Exosomes

Therapeutic MSC-exosome was first described by Lai et al. in 2010 [59]. Once this critical function was reported, the interest to unearth the interactions between MSC-exosomes with microenvironmental niche underwent a new upsurge [60, 67, 77]. Among these intricate and complex relationships in which MSC-exosomes participate, the key pathological processes such as inflammation, oxidative stress, antitumor effect, and regenerative nature have drawn wide attention (Figure 2) [2, 12, 28, 35, 46].

4.1. MSC-Exosomes and Immunomodulation. Accumulating evidence demonstrated that MSC-exosomes could exert powerful immunomodulatory effects by delivering biological factors such as cytokines (interleukin- (IL-) 2, IL-6, IL-1β, IL-10, tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), interferon (IFN)-γ, etc.), and chemokines (C-C motif ligand (CCL)-2, CCL-3, CCL-7, C-X-C motif chemokine (CXC)-12, CXC-14, etc.) [34, 78–80] (Figure 2(a)). In addition, these bioactive cargoes efficiently interacted with various kinds of immune cells including macrophages, natural killer (NK cells), T cells, B cells, regulatory T cells (Tregs), and dendritic cells (DCs) [28, 78]. Surprisingly, MSC-exosomes could also constitutively alter the immunomodulatory mechanisms in dependence of milieu they exposed to. That is, when exposed to the low levels of inflammatory cytokines, MSC-exosomes obtained proinflammatory function and stimulated activation of immune cells [78]. On the contrary, in circumstances where immune responses were excessive, they adopted anti-inflammatory phenotype and secreted immunosuppressive factors that

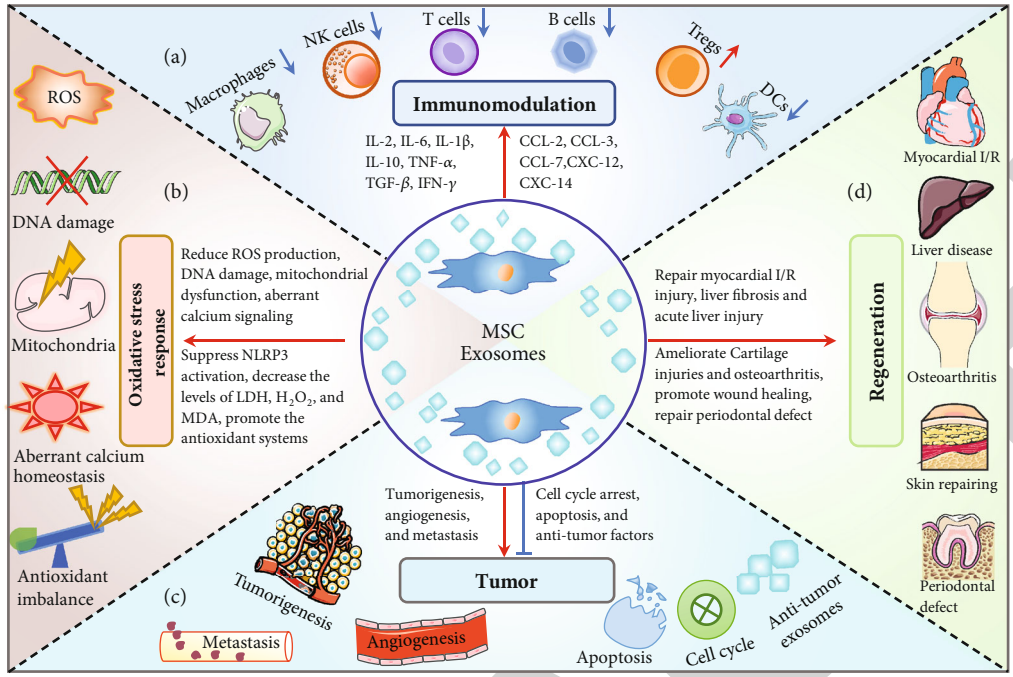

Figure 2: The biological mechanisms of MSC-exosomes. (a) Immunomodulatory effects of MSC-exosomes. (b) Reactions of MSC-exosomes in response to oxidative stress. (c) Interactions between tumor cells and MSC-exosomes. (d) Applications of MSC-exosomes in regenerative medicine. IL: interleukin; TGF-β: transforming growth factor beta; IFN-γ: interferon γ; CCL: C-C motif ligand; CXC: C-X-C motif chemokine; NK cells: natural killer cells; Tregs: regulatory T cells; DCs: dendritic cells; ROS: reactive oxygen species; I/R: ischemia/reperfusion.

decreased generation of proinflammatory cells and inhibited function of effector immune cells [58, 78, 79, 81].

4.2. MSC-Exosomes and Oxidative Stress. Oxidative stress, as was reflected by levels of reactive oxygen species (ROS), mitochondrial dysfunction, and aberrant calcium signaling [82], has been recognized as a contributing factor in tumorigenesis and involved in the progression of multiple diseases including myeloid leukemia, abnormal hematopoiesis, colon inflammation, and liver fibrosis [34, 79, 83] (Figure 2(b)). Nowadays, MSC-exosomes, as a cell-free strategy, have attracted considerable attention due to their robust antioxidative capacities [34, 35, 82, 84]. They were reported to reduced ROS generation, DNA damage, aberrant calcium signaling, and mitochondrial changes via regulation of the NRF2 system in oxidative stress-induced skin injury [35]. They were discovered to ameliorate intervertebral disc degeneration by suppressing NLRP3 inflammasome activation and delivering mitochondrial proteins to restore the damaged mitochondria of nucleus pulposus cells [85]. In addition, they were found to promote the recovery of hepatic oxidant injury and apoptosis in vitro and in vivo by delivery of glutathione peroxidase 1. Despite these powerful antioxidative and anti-inflammatory effects by MSC-exosomes, however, their potential therapeutic agents in hematological diseases are still unclear and warrant further explorations.

4.3. MSC-Exosomes and Tumor Therapy. MSC-exosomes, two-edged sword in cancer therapy, play dual effects on tumor cells. Several studies suggested that MSC-exosomes performed as mediators in the tumor niche and promoted tumorigenesis, angiogenesis, and metastasis (Figure 2(c)). In contrast, other reports supported the tumor-suppressing effects by inhibiting cell cycle and inducing apoptosis [86]. In addition, when MSCs were pretreated with antitumor factors, a significant tumor-suppressing effect was obtained in MSC-exosomes [67, 87]. In this regard, MSC-derived exosomes are poised to become the next generation of smartly engineered delivery vehicles for precision medicine [20, 87, 88].

4.4. MSC-Exosomes and Regeneration. The first reported therapeutic efficacy of MSC-exosomes was to mediate cardioprotection in a mouse model during myocardial ischemia/reperfusion (I/R) injury [59]. Smaller and less complex than their parent stem cells, MSC-exosomes were potent enough to be used for cell-free regeneration of liver fibrosis and acute liver injury [26]. Besides, these exosomes were also proved to accelerate cartilage regeneration and osteoarthritis repair [89]. By virtue of reparative and regenerative properties, diabetic wound resulted in a significantly accelerated wound closure rate with MSC-exosomes treatment [90]. Furthermore, MSC exosome-loaded collagen sponge was reported to promote periodontal regeneration in a periodontal defect model [91]. As was shown in Figure 2(d), these biotherapeutics, primarily through the transfer of MSC-exosomes cargoes, could perhaps provide novel insights for the treatment of hematological diseases.

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Table 1: MSC-exosomes in hematological diseases.

Disease	MSC sources	Exosomal cargo	Disease model	Biological effect	Ref.
Refractory GVHD	Human BM-MSCs	NM	Clinical case	Reduced proinflammatory cytokine and improved clinical GvHD symptoms	[37]
aGVHD	Immortalized human embryonic stem cell-derived MSCs	NM	Mouse GVHD model	Enhanced Treg production, alleviated GVHD symptoms, and increased survival by APC	[95]
aGVHD	Human BM-MSCs	miR-125a-3p	Mouse GVHD model	Prolonged the survival of mice with aGVHD and reduced the pathologic damage by suppressing the functional differentiation of T cells from a naive to an effect or phenotype	[27]
aGVHD	Human UC-MSCs	NM	Mouse GVHD model	Lowered the number of $CD3^+CD8^+$ T cells; reduced levels of IL-2, TNF-α, and IFN-γ; increased the ratio of $CD3^+CD4^+/CD3^+CD8^+$ T cells; and rose serum levels of IL-10	[96]
GVHD	Human UC-MSCs	TGF-β, IFN-γ, IDO, IL-10	In vitro cell experiment	Promoted PBMCs to differentiate into Tregs via TGF-β and IFN-γ	[94]
cGVHD	Human BM-MSCs	NM	Mouse chronic GVHD	Blocked Th17 differentiation and improved the Treg phenotype	[97]
cGVHD	Human UC-MSCs	NM	Mouse chronic GVHD	Prevented skin fibrosis in the cGVHD mouse model by suppressing the activation of macrophages and B cell immune response	[98]
MM/lymphoma/ leukemia	Young and elderly healthy donor BM-MSCs	NM	In vitro cell experiment	Antitumor effect existed in the supernatant and not in exosomes; the antiangiogenesis effect depends on the age of donors	[43]
MM	MM-derived BM-MSCs	miRNA-15a, IL-2, CCL-2, fibronectin	Mouse MM model	MM patient-derived BM-MSC exosomes promoted MM tumor growth while normal-derived exosomes inhibited the growth of MM cells	[42]
MM	Human BM-MSCs and mouse BM-MSCs	MCP-1, IP-10, SDF-1	In vitro and in vivo MM model	Favored MM cell proliferation, migration, and survival and induce drug resistance to bortezomib	[100]
MM	Normal donors and MM BM-MSCs	NM	In vitro cell experiment	Decreased cells viability, proliferation, migration, and translation initiation with exosomes from normal donor BM-MSCs, whereas MM MSC-exosomes increased	[101]
MM	Old and young MM-derived BM-MSCs	miR-340	In vivo model of hypoxic BM in MM	Inhibited MM-induced angiogenesis with exosomes from young BM-MSCs, and miR-340 inhibited angiogenesis in endothelial cells	[103]
MM	MM and normal tissue-derived MSCs	LINC00461	In vitro cell experiment	LINC0046 was highly expressed in MSC exosomes and enhanced MM cell proliferation	[102]
MM	Bortezomib-resistant or bortezomib-sensitive patient MSCs	lncPSMA3, PSMA3-AS1	U266-luc^+ xenograft models	Exosomal lncPSMA3-AS1 mediated resistance to proteasome inhibitors by regulating the stability of PSMA3	[30]

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Table 1: Continued.

Disease	MSC sources	Exosomal cargo	Disease model	Biological effect	Ref.
AML	Human BM-MSCs	S100A4	In vitro cell experiment	Upregulated S100A4 and driven proliferation, invasion, and chemoresistance of leukemia cells	[106]
AML	Human BM-MSCs	TGFB1, miR-155, miR-375	Clinical sample analysis	Released TGFB1, miR155, and miR375 to mediate extrinsic chemoresistance within the niche in AML	[33]
AML	HD or newly diagnosed AML patient BM-MSCs	miR-26a-5p, miR-101-3p, miR-23b-5p, miR-339-3p, miR-425-5p	Clinical sample analysis	Identified candidate miRNAs that provide new insights regarding leukemogenesis and new treatment strategies	[39]
CML	Human UC-MSCs	NM	In vitro cell experiment	Enhanced the sensitivity of K562 cells to imatinib (IM) via activation of the caspase signaling pathway	[105]
CML	Human BM-MSCs	miR-15a	CML xenograft tumor model	Inhibited CML cell proliferation, decreased their sensitivity to IM, and promoted IM resistance	[32]
CLL	Human BM-MSCs	NM	In vitro cell experiment	Rescued leukemic cells from spontaneous or drug-induced apoptosis, enhanced their migration, and induced gene expression modifications	[40]
Hodgkin lymphoma	MSC cell lines	ADAM10	In vitro cell experiment	Induced release of cytokines, like TNFα, sCD30, or CD30 shedding by HL cells	[31]
MDS	HD and MDS patient BM-MSCs	miR-10a, miR-15a	In vitro cell experiment	MDS BM-MSC-derived cargoes overexpressed miR-10a and miR-15a and enhanced cell viability and clonogenic capacity of $CD34^+$ cells	[41]

NM: not mentioned; HD: health donor; aGVHD: acute GVHD; UC-MSC: umbilical cord MSC; MDS: myelodysplastic syndrome; miRNAs: microRNAs; MCP-1: monocyte chemoattractant protein 1; IP-10: interferon-inducible protein 10; SDF-1: stromal cell-derived factor 1; imatinib: IM.

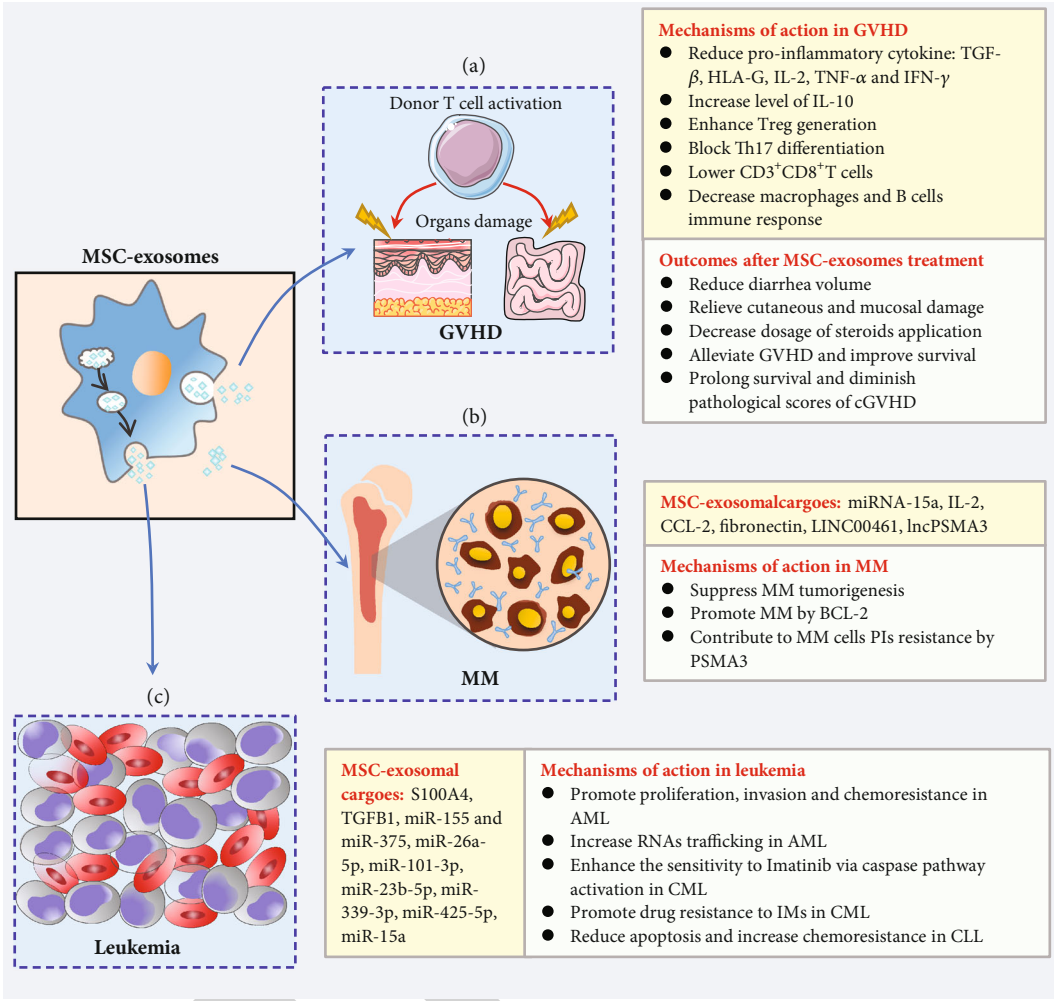

Figure 3: Schematic diagram of molecular mechanisms MSC-exosomes in hematological diseases. (a) The action of MSC-exosomes and subsequent clinical outcomes in GVHD. (b) A brief outline of exosomal cargoes and underlying mechanisms of MSC-exosomes in MM. (c) Exosomal loadings and potential effects of MSC-exosomes in the diseases of AML, CML, and CLL. HLA-G: human leukocyte antigen-G; cGVHD: chronic GVHD; MM: multiple myeloma; AML: acute myeloid leukemia; CML: chronic myeloid leukemia, CLL: chronic lymphocytic leukemia; PIs: proteasome inhibitors; IM: imatinib.

5. Mechanisms of MSC-Exosomes in Hematological Diseases

Currently, MSC therapies are underway, and their properties of multipotentiality and low immunogenicity have made them promising cell-based strategies for various types of clinical applications [27, 28, 42]. However, their beneficial effects have been hampered due to the capacity that unexpectedly differentiate or uncontrollably grow in the hosts [92]. Indeed, recent data have implied that MSCs exerted their therapeutic functions in a paracrine by releasing exosomes rather than in a cellular manner [93]. In the last decade, the secreting activity of MSCs has been widely investigated. MSC-exosomes, as an acellular product, are reported more superior to their parent stem cells in that they are smaller, less complex, and immunogenic and thus easier to produce and store [81]. Moreover, the contents of MSC-exosomes under different incubation conditions could be artificially altered, orchestrating more accurate immune modulation network [6, 94]. In the following section and in Table 1, we particularly examined the evidence to date for therapeutic potential and relevant mechanisms of MSC-exosomes in several important hematological diseases (Figure 3) and discussed some of the future challenges to their successful clinical translation.

5.1. MSC-Exosomes in GVHD. Various studies have demonstrated the unique immunomodulatory potential and extensive tissue repair ability of MSC-exosomes in disease of GVHD (Figure 3(a)). In a clinical study to treat refractory GVHD, Kordelas and colleges showed that MSC-exosomes induced high quantities of the anti-inflammatory molecules IL-10, TGF-β, and human leukocyte antigen-G (HLA-G) [37]. Shortly after the clinical administration of MSC-exosome therapy, GVHD symptoms could be significantly improved, and the dosage of the steroids could be remarkably reduced. Another research was in a mouse GVHD model, and Zhang et al. reported that MSC-exosomes could

generate Tregs by activating T cells through an APC-mediated pathway [95]. Their daily observations and disease index assessments showed that systemic administration of MSC-exosomes alleviated GVHD symptoms and prolonged overall survival. Findings of Fujii and coworkers indicated that the numbers of $CD4^+$ and $CD8^+$ T cells were decreased, the differentiation of naive T cells to an effector phenotype was suppressed, and the pathologic damage of GVHD-targeted organ was alleviated in MSC-exosome-treated GVHD mice while the normal fibroblasts-derived exosome treatment did not ameliorate the pathological manifestations [27], which mean the unique immunoregulatory function of MSC-exosomes.

Until now, only few studies indicated that human umbilical cords derived MSC-exosomes (UC-MSC-exosomes) as an alternative in the prophylaxis of GVHD. One of researches was conducted in the mouse model of acute GVHD (aGVHD) with the treatment of UC-MSC-exosomes [96]. They found that UC-MSC-exosome intervention significantly lowered frequencies of $CD3^+CD8^+$ T cells and reduced levels of IL-2, TNF-α, and IFN-γ, but elevated the serum IL-10. Another research by Zhang et al. demonstrated that TGF-β and IFN-γ incubated UC-MSC-exosomes possessed more potent immune regulation property via promoting differentiation into Tregs and increasing a variety of cytokines such indoleamine 2,3-dioxygenase (IDO) [94].

A chronic GVHD (cGVHD) mouse model revealed that MSC-exosomes effectively ameliorated fibrosis in the skin, lung, and liver and exhibited potent immunomodulatory effects via the inhibition of IL-17-expressing Th17 cells and induction of IL-10-expressing Tregs [97]. Corresponding to their in vivo experiment, MSC-exosomes in vitro blocked Th17 differentiation and improved the Tregs phenotype, further confirming the regulatory effects on GVHD effector T cells [97]. Consistent with the above conclusions, Guo et al. corroborated that MSC-exosomes treatment reduced the cGVHD scores, alleviated fibrosis of the skin in sclerodermatous cGVHD mice, reduced the macrophage infiltration, decreased TGF-β and smad2 production, and suppressed the activation of B cells immune response in the skin [98].

Collectively, these researches suggest that the administration of MSC-exosomes represents a new, cell-free therapeutic approach for attenuation of GVHD and immune disorders. It expands the horizon for utility of MSC-exosomes and provides a new insight for GVHD as well as other immune system imbalance.

5.2. MSC-Exosomes in MM. Despite therapeutic advances over the past decade with proteasome inhibitors and immunomodulatory drugs, however, MM remains incurable especially in relapsed and/or refractory patients [99]. As delivery and communication cargoes, MSC-exosomes play a generally unrecognized yet significant role in MM development and progression (Figure 3(b)).

The results of high-throughput antibody-based protein array revealed the specifically higher levels of IL-6, CCL2, junction plakoglobin, and fibronectin in MM-derived MSC-exosomes, suggesting that exosomes behaved as vesicles and selectively transported certain proteins to the recipient cells [42]. Another interesting research was conducted by Wang and colleges [100]. They depicted that MSCs and MM cells could communicate with each other and exchange cytokines through exosome secretion and uptake. By carrying the selective cytokines such as monocyte chemoattractant protein 1 (MCP-1), interferon-inducible protein 10 (IP-10), and stromal cell-derived factor 1 (SDF-1), MSC-exosomes could favor MM cell proliferation, migration, and survival and induce drug resistance to bortezomib [100], which revealed a novel agent for drug resistance in MM. To our best knowledge, there are striking differences in accordance with normal or pathological source of MSC-exosomes. Just as Dabbah et al. proved, MM-derived MSC-exosomes increased MM cells viability, proliferation, migration, and invasion, whereas the normal derived group decreased [101]. Interestingly, these differences could be markedly attenuated by inhibiting MAPK signaling.

Emerging evidence documented that MSCs released large amounts of exosomes loaded with bioactive components include noncoding RNAs (ncRNAs) which contributed to disease initiation, evolution, and treatment [61, 65, 102]. Umezu et al. provided a good example [103]. They investigated the therapeutic potential of MSC-exosomes derived from young and older donors using an in vivo MM model. They found that exosomal miRNA expression profile was different especially with preferentially expressed miR-340. They finally made a conclusion that direct transfection of miR-340 to the older MSC-exosomes inhibited angiogenesis via the hepatocyte growth factor/c-MET (HGF/c-MET) signaling pathway in endothelial cells. A similar research was drawn by Roccaro and coworkers [42]. They unraveled the profile of miRNAs and proteins between MM and normal MSC-exosomes and indicated that exosomal miRNAs could mediate epigenetic transfer from MSCs to MM cells [42]. They also demonstrated that miR-15a in MSC-exosomes was significantly increased in normal versus MM patients, suggestive of a tumor-suppressive role of miR-15a. Another in vitro experiment confirmed that LINC00461, a sponge for miR-15a/16, was highly expressed in MSC-exosomes, which promoted MM tumorigenesis via dramatically decreasing the expression of BCL-2 [102]. Similarly, results from Xu et al. indicated that exosomes mediated lncPSMA3-AS1 transfer from MSCs to MM, which contributed to proteasome inhibitors (PIs) resistance by regulating the stability of PSMA3 [30]. They also provided in vitro and in vivo evidence that interference with exosomal RNAs could serve as a promising approach to overcome PI resistance in MM.

5.3. MSC-Exosomes in Leukemia. Cumulative findings have demonstrated that MSC-exosomes, working as an important element of tumor microenvironment niche, play a vital role in leukemia cell proliferation and drug resistance (Figure 3(c)) [11, 104]. According to published papers, evidence underscored the hypothesis that the mechanisms of MSCs, at least in leukemia, were attributed mainly to secreting function by exosomes shuttle [39, 40, 105]. With regard

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to AML, Lyu and colleagues recently highlighted the profound impact of MSC-exosomes on leukemia cells [106]. They examined the efficacy after treatment with MSC-exosomes and found that MSC-exosomes functionally promoted the proliferation, invasion, and chemoresistance of tumor cells via upregulation of S100A4, a typical member of the S100 family of calcium-binding proteins [106]. Releasing risk factors by cargoes of MSC-exosomes, including TGFB1, miR-155, and miR-375, was another underlying mechanism for chemoresistance within the niche of AML [33]. Study using next generation sequencing found five candidate miRNAs that were differential packaged in MSC-exosomes, including significantly increased miR-26a-5p and miR-101-3p and strikingly decreased miR-23b-5p, miR-339-3p, and miR-425-5p, which indicated that miRNAs from AML-derived MSC-exosomes might be implicated in leukemogenesis [39]. Given that the rapid pace of sequencing technologies development and their applications, RNAs profiling will provide new insights for AML treatment by identifying the differentially expressed molecules.

Despite patients with CML generally exhibited remarkable efficacy of tyrosine kinase inhibitors (TKIs), there was a subgroup of patients who were resistant and/or intolerant to TKIs. Studies supported the notion that exosomes were extensively involved in drug resistance [23]. Liu et al. clarified that human UC-MSC-exosomes alone had no effect on K562 cell viability and apoptosis of tumor cells, while promoted imatinib-induced cell viability inhibition and apoptosis via activation of the caspase signaling pathway [105]. In contrast, another similar research yielded incredibly different conclusions that human BM MSC-exosomes could inhibit the proliferation of K562 cells via miR-15a and arrest cell cycle in vitro. Obviously, they got the contradictory conclusion that MSC-exosome administration resulted in drug resistance by promoting the CML cells proliferation and decreasing the sensitivity to TKIs [32]. Since interaction between MSC-exosomes and CML cells has remained controversial, therefore, indepth theoretical modeling needs to be further established.

Chronic lymphocytic leukemia (CLL) is the most prevalent leukemia in the West. However, a literature search revealed that the role of MSC-exosomes in CLL occurrence and progression was largely unexplored. Crompot and coinvestigators discovered a decrease of leukemic cells spontaneous apoptosis and an increase in their chemoresistance to several drugs when tumor cells cocultured with MSC-exosomes in vitro [40]. Intriguingly, their research indicated that patients derived MSC-exosomes induced a higher migration capacity and a stronger gene modification of CLL compared to healthy donors. These findings indicated an interesting direction for MSC-exosome therapies in CLL under physiological and pathological conditions.

5.4. MSC-Exosomes and Other Hematological Diseases. Findings demonstrated the multifunctional roles of in leukemic progression and GVHD treatments [23, 32, 105] (Table 1). They were also proved to be crucial for the hematopoietic system and other hematological diseases [36]. As demonstrated by Wen and coworkers, MSC-exosomes rescued radiation damage to the marrow hematopoietic cells by attenuating DNA damage and apoptosis and recovered homeostasis by stimulating normal marrow cells proliferation [36]. With regard to lymphoma, Gladkova et al. came to a conclusion that the antiangiogenesis effect mainly depended on soluble factors existing in supernatant rather than in of MSC-exosomes [43]. Nevertheless, another similar finding showed that MSC-exosomes induced release of cytokines such as TNF-α and CD30 shedding from Hodgkin lymphoma (HL) cells, which potentially interfered with host immune surveillance or immunotherapy [31]. Given that exosomes could provide a platform of intercellular communication through miRNA delivery, Muntion and coworkers documented that BM-MSC released exosomes with different expression profiles in MDS patients compared with health donors [41]. The loaded miR-10a and miR-15a could be incorporated into hematopoietic progenitors and consequently exhibited higher cell viability and clonogenic capacity in MDS patients [41].

6. Conclusion

A number of biologic functions of MSCs have been mediated in a paracrine manner by secreting exosomes. MSC-exosomes, as a biological acellular product, have a number of advantages over their counterpart MSCs due to their small size, lack of toxicity, and low immunogenicity. Considerable evidence has pointed that their biological potential is attributed to the action involving in alleviating inflammation, repressing oxidative stress, balancing homeostasis, regulating antitumor effects, and repairing the impaired tissue cells. MSC-exosomes exert their intricate effects via transferring materials include cytokines, proteins, mRNAs, and miRNAs in hematological malignancies. Despite the intrinsic capability to transfer, the components of cargoes can differ seriously from disease suppression to disease promotion, which rely heavily on the microenvironment niche, and they exist in (Table 1). Nowadays, high-throughput sequencing is widespread, and engineering strategies to modify exosomes are on the way [107, 108], which open a novel scenario for accurate utilization of MSC-exosomes in practice. Accordingly, future precision medicine for MSC-exosomes in hematological diseases is perhaps to evaluate molecular mechanisms, to engineer for the next-generation delivery system, and to promote translation of basic science to widespread clinical use.

Conflicts of Interest

All authors declare no conflicts of interest.

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Retraction

Retracted: Exosome: Function and Application in Inflammatory Bone Diseases

Oxidative Medicine and Cellular Longevity

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope
(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
(4) Inappropriate citations
(5) Incoherent, meaningless and/or irrelevant content included in the article
(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] Y. Hu, Y. Wang, T. Chen, Z. Hao, L. Cai, and J. Li, "Exosome: Function and Application in Inflammatory Bone Diseases," *Oxidative Medicine and Cellular Longevity*, vol. 2021, Article ID 6324912, 17 pages, 2021.

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Review Article

Exosome: Function and Application in Inflammatory Bone Diseases

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In the skeletal system, inflammation is closely associated with many skeletal disorders, including periprosthetic osteolysis (bone loss around orthopedic implants), osteoporosis, and rheumatoid arthritis. These diseases, referred to as inflammatory bone diseases, are caused by various oxidative stress factors in the body, resulting in long-term chronic inflammatory processes and eventually causing disturbances in bone metabolism, increased osteoclast activity, and decreased osteoblast activity, thereby leading to osteolysis. Inflammatory bone diseases caused by nonbacterial factors include inflammation- and bone resorption-related processes. A growing number of studies show that exosomes play an essential role in developing and progressing inflammatory bone diseases. Mechanistically, exosomes are involved in the onset and progression of inflammatory bone disease and promote inflammatory osteolysis, but specific types of exosomes are also involved in inhibiting this process. Exosomal regulation of the NF-κB signaling pathway affects macrophage polarization and regulates inflammatory responses. The inflammatory response further causes alterations in cytokine and exosome secretion. These signals regulate osteoclast differentiation through the receptor activator of the nuclear factor-kappaB ligand pathway and affect osteoblast activity through the Wnt pathway and the transcription factor Runx2, thereby influencing bone metabolism. Overall, enhanced bone resorption dominates the overall mechanism, and over time, this imbalance leads to chronic osteolysis. Understanding the role of exosomes may provide new perspectives on their influence on bone metabolism in inflammatory bone diseases. At the same time, exosomes have a promising future in diagnosing and treating inflammatory bone disease due to their unique properties.

1. Introduction

Bone homeostasis is a balance of osteoblasts and osteoclasts constantly acting on the bone to renew the body's bone mass. Chronic inflammation caused by various stressors can to disrupt this delicate balance between osteoblasts and osteoclasts by secreting various inflammatory factors, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and prostaglandin E2 (PGE2), leading to disturbances in bone metabolism [1]. These osteolytic lesions caused by chronic aseptic inflammation include aseptic loosening of the prosthesis [2], osteoporosis [3, 4], and rheumatoid arthritis [5]. Aseptic loosening around the prosthesis is an outstanding representative of these disorders.

Exosomes, as transmitters of intercellular information, may be an emerging target for our exploration of the pathogenesis of inflammatory bone diseases and their therapeutic targets. Extracellular vesicles include four subgroups, namely, exosomes, microparticles, apoptotic vesicles, and cancer vesicles [10]. The current research hotspot is the subgroup of exosomes. Exosomes are disc-shaped vesicles that contain polysaccharides, lipids, metabolites, RNA (microRNA and lncRNAs), DNA, and specific proteins [11]. With a diameter of 40–100 nm, these tiny vesicles can act as signaling molecules to other cells, thereby altering the function of other cells [12]. These findings have inspired a number of studies on exosomes. Exosomes serve as an essential mediator of intercellular communication, altering the phenotype of target cells by delivering cargo into the cytoplasm. Given the selective loading of cargo, cargo composition in exosomes may differ from the tissue or cells from which they originate [13]. As in other cell types, macrophages deliver inflammatory signals by extracellular vesicles. Research

increasingly shows that exosomes are involved in a variety of pathological processes, including chronic inflammation [14]. Based on the function of exosomes in delivering specific proteins and nucleic acids to specific target cells, it is reasonable to assume that exosomes could also play an essential role in intercellular communication in inflammatory bone diseases. Macrophage function is closely related to inflammation that is closely related to bone metabolism. Reversing inflammatory osteolysis by designing the effects of different types of exosomes on macrophage function and bone metabolism, and ultimately cell-free therapy, is challenging, which will also provide a new perspective for researchers. A better understanding of the pathogenesis of inflammatory bone disease has important implications for the design of novel therapeutics for inflammatory bone disease. In this comprehensive review, we highlight the effect of exosomes on inflammatory bone disease through their roles in inflammation and bone metabolism. Enhanced osteoclast activity and diminished osteoblast activity are the two main aspects of bone metabolism disorders in inflammatory bone diseases. However, the body also self-regulates and produces protective exosomes to counteract this process. Finally, we discuss the possible applications of exosomes in inflammatory bone diseases in clinical diagnosis and treatment.

2. Overview of Exosomes

Exosomes are extracellular vesicles from the endosomal pathway in the nanometer diameter range that can be secreted by most cells under physiological or pathological conditions by cytosolic exocytosis [15]. Exosomes are widely found in various body fluids of living organisms, such as blood, urine, saliva, ascites, and bile. The secretory pathway of exosomes has been extensively studied since its discovery, and exosomes from different cells have similar effects to their parent cells. Exosomes are involved in many physiological and pathological processes, including tissue damage and repair responses, by coordinating the communication between different cell types. They act as vectors between different cell types that transfer nucleic acids, proteins, or lipids to target cells, causing changes in the phenotype and function of the target cells [16]. More than 41,860 proteins, 7,540 RNAs, and 1,116 lipid molecules have been identified in exosomes [17]. The formation of exosomes involves the following steps: first, inward budding of endosomal membranes to generate multivesicular bodies (MVBs); then, MVBs fuse with the plasma membrane and release their luminal vesicles to form exosomes [18]. Exosomes can travel throughout the body via the circulatory system and cross the blood-brain barrier and other tissues to be taken up by target cells (Figure 1). Extracellularly, signals are delivered from exosomes to recipient cells in three ways: receptor-ligand binding, membrane fusion, or phagocytosis [19]. Exosome secretion occurs naturally in organisms, and stress and inflammatory signals can regulate the processes involved [20].

Exosomes may play an essential role in sterile inflammation, including prosthesis loosening, because they can modulate immunity by transmitting inflammatory signals and regulating macrophage differentiation [21]. A growing number of studies emphasize the role of exosomes in inflammation-associated intercellular communication. Currently, only a few studies have been conducted on exosomes associated with aseptic loosening compared with other aseptic inflammatory conditions of the bone. The limited studies may be because experimental models of aseptic loosening are more complex and challenging to establish than those of other disease pathogenesis. However, it may share similar signaling cascades with diseases, such as rheumatoid arthritis and osteoarthritis, although such diseases are mediated by different triggers. Some of the pathogenic processes associated with the development of inflammatory bone diseases in exosomes include the delivery of miRNAs, lncRNAs, inflammatory cytokines, chemokines, proteases, and other proteins; activation of macrophages; activation of Toll-like receptors; intercellular communication; and degradation of the extracellular matrix.

3. Exosomes and Macrophage Polarization

Macrophage polarization is closely associated with inflammatory bone disease. Excessive numbers of macrophages or M1 polarization can induce delayed bone healing and chronic inflammation. Chronic inflammation leads to the production of various inflammatory factors that cause progressive osteolysis, as well as inhibit bone formation. Macrophages, as regulators of inflammation, are central to the pathogenesis of inflammatory bone diseases due to aseptic inflammation. Macrophages are important coordinators of immune activity and homeostasis in the body and are involved in the elimination of foreign substances, relief of inflammation, and tissue repair. At present, macrophages are recognized to play an important role in the etiology of inflammatory bone diseases. Macrophages in areas of inflammatory tissue are derived from monocytes in the bloodstream. Activated macrophages can be categorized into the M1 phenotype (classically activated macrophage phenotype) and the M2 phenotype (alternative activated macrophage phenotype) [22]. In the presence of interferon γ (IFN-γ) and lipopolysaccharide (LPS), macrophages polarize toward the M1 phenotype. Nevertheless, in the presence of interleukin-4 (IL-4), another well-known cytokine produced by T cells, macrophages polarize to the M2 phenotype. Macrophage polarization induced by various stressors, such as abrasive debris in aseptic loosening, favors the M1 phenotype. M1 phenotype macrophages play a defensive role when interacting with biological materials. They perform phagocytic functions to remove pathogens and debris from injury sites and perform proinflammatory functions by secreting TNF-α, IL-1, and IL-6 [23]. M2 phenotype macrophages exert anti-inflammatory effects and secrete interleukin-10 (IL-10) [24]. The dynamic balance between M1-like and M2-like macrophages strictly controls the outcome of the disease. Macrophage polarization toward the M1 phenotype is one of the most important manifestations in the development of aseptic bone inflammation. Nuclear factor-κB (NF-κB) is a key transcription factor in macrophages that regulates macrophage polarization. An increasing number of

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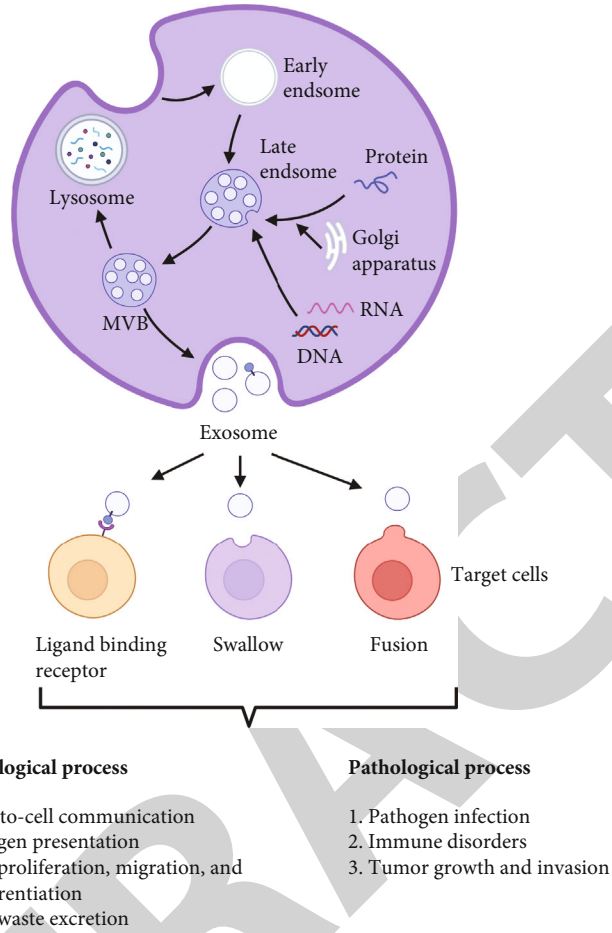

Figure 1: Process of exosome production and delivery. The plasma membrane internalizes to form early endosomes and gradually forms late endosomes. With the entry of intracytoplasmic proteins, lipids, DNA, and RNA cargoes, late endosomes produce a large number of intraluminal vesicles (ILV) inside the endosome, which gradually evolve into multivesicular bodies (MVBs). MVB can be degraded by the action of lysosomes to form lysosomes or fuse with the plasma membrane to release ILV further outside the cell to form exosomes. Exosomes released into the extracellular compartment enter the recipient cell in three ways: receptor-ligand binding, membrane fusion, and phagocytosis.

studies have found that exosomes can alter the polarization phenotype of macrophages by stimulating the NF-κB signaling pathway and a variety of other pathways (Figure 2).

3.1. Exosomes Activate Toll-Like Receptors. Innate immune cells use their pattern recognition receptors to detect pathogen-associated molecular patterns and damage-associated molecular patterns, which play a decisive role in host defense against invading pathogens [8]. As a characteristic representative of pattern recognition receptors, the Toll-like receptor (TLR) is known to identify endogenous and infectious stress that triggers inflammation and aids in adaptive immune responses. It is intriguing that exosomes can also activate Toll-like receptors to trigger inflammatory responses. Plasma exosomes from patients with rheumatoid arthritis can activate TLR4, and this activation mechanism is similar to that of TLR4 activation by LPS [25]. Further studies showed that oxidized phospholipids on exosome membranes are responsible for the stimulation of TLR-4 [25]. TLR4 can recruit MyD88 when it is activated. The binding of TLR4 and MyD88 phosphorylates IRAK4, which in turn phosphorylates IRAK1. The tight packing of IRAKs activates their potential kinase activity, driving autophosphorylation and subsequent recruitment of the E3 ubiquitin ligase TRAF6. TRAF6 activates the kinase TAK1, which stimulates IκB kinase- (IKK-) mediated NF-κB and mitogen-activated protein kinase- (MAPK-) mediated AP-1 transcriptional responses [26]. In a nutshell, the MyD88-dependent pathway leads to activation of NF-κB and activator protein-1 (AP-1) that promote the secretion of proinflammatory cytokines, such as TNF-α, IL-1, and IL-12. Exosomes act as an endogenous danger signal induced by oxidative stress and play a vital role in the onset and development of inflammatory bone diseases.

The process of aseptic loosening also involves the activation of Toll-like receptors. For example, TLR1/TLR2 heterodimer can be activated by UHMWPE, while TLR4 can be activated by cobalt or nickel ions [27, 28]. However, no further studies have been done to investigate whether exosomes are involved in this process in aseptic loosening. Recent studies have found that TLR can be transmitted between immune cells via exosomes and can increase the

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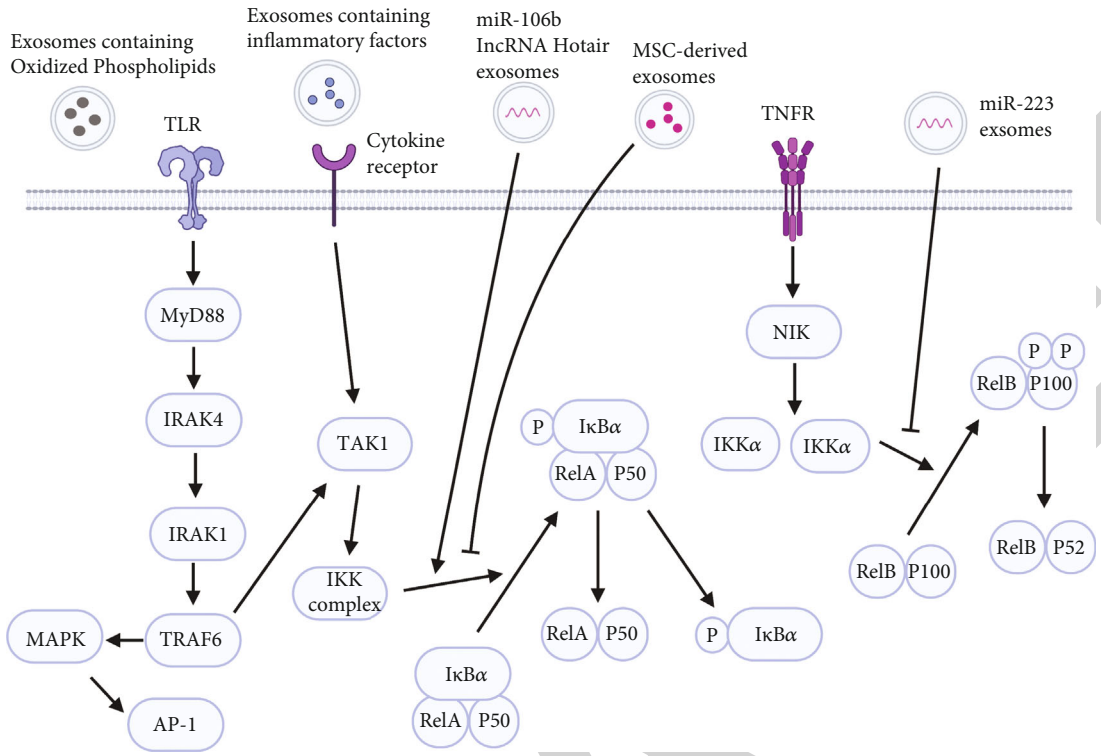

Figure 2: Effect of exosomes on macrophage differentiation. Toll-like receptor recruitment MyD88 mediates the activation of TNF receptor-associated factor-6 (TRAF6). TRAF6 promotes AP-1 signaling or NF-κB activity via the IκB kinase (IKK) complex. The IKK complex mediates the phosphorylation and subsequent degradation of IκBα, while the IKKα dimer mediates the phosphorylation and degradation of p100, both of which can produce active NF-κB. Exosomes containing oxidized phospholipids activate Toll-like receptors and promote the production of active NF-κB. Inflammatory cytokines in exosomes bind to cytokine receptors, triggering the classical NF-κB pathway, activating TAK1, and inducing NF-κB activity. Exosomes containing miR-106b or lncRNA Hotair and those that are mesenchymal stem cell (MSC) derived promote IκBα phosphorylation. Exosomes containing miR-223 inhibit the phosphorylation of p100 by IKKα.

responsiveness of recipient cells to LPS [29]. These findings show that exosomes play an essential role in the activation of Toll-like receptors. Whether exosomes have other roles on Toll-like receptor activation in inflammatory bone diseases, such as whether exosomes can activate other subtypes of Toll-like receptors and the conditions of activation, remains to be further explored.

3.2. Exosomes Contain a Variety of Inflammatory Cytokines. Macrophage-derived exosomes represent a significant fraction of the exosomal component of the blood. Several studies have found that exosomes produced by immunocytes, such as macrophages and dendritic cells, contain various proteins without N-terminal signaling peptides, including chemokines and inflammatory cytokines [30]. These exosomal components can influence the progression of inflammatory bone diseases, but their effects are not dependent on the exosome structure. In other words, these inflammatory and chemokine components are present in exosomes, are widely distributed in the body, and are involved in the process of inflammatory bone diseases. Inflammatory cytokines promote NF-κB expression through activation of the NF-κB signaling pathway by cytokine receptors to regulate macrophage polarization.

Exosomes isolated from patients with rheumatoid arthritis contained a membrane-bound form of tumor necrosis factor-α, which activates NF-κB and leads to the induction of matrix metalloproteinase-1 (MMP-1) [31]. Inflammatory cytokines, such as TNF-α and IL-1β, can cause an inflammatory cascade around the prosthesis. TNF-α is the major anti-M2 inflammatory cytokine that impedes M2 macrophage production by acting directly on unactivated macrophages and affecting IL-13 production and AMPK phosphorylation in other cell types [32]. TNF-α also activates macrophages to release other inflammatory cytokines, such as IL-6 and IL-1β, which promote inflammatory responses. However, the membrane-bound form of TNF-α should be cleaved into a soluble form to promote osteoclast formation. IL-1β, a critical inflammatory cytokine in the inflammatory cascade response, is also secreted in exosomal form as an essential adjunct to its output [33–35]. IL-1β is not only released by cells during the fusion of secretory lysosomes with the plasma membrane but is also secreted by exosomes [36]. When ATP binds to P2X7R in the exosome, IL-1β is released from the exosome to regulate the inflammatory response [37]. IL-1β is considered one of the most important family members of the interleukin family with intense proinflammatory activity by stimulating the production of various proinflammatory mediators, such as cytokines, chemokines, and matrix metalloproteinases (MMPs) [38]. IL-1β and IL-6 act synergistically and activate each other's expression, with positive feedback leading to the continued progression of

chronic inflammation. Production of monocyte chemoattractant protein-1 (MCP-1), IL-6, IL-8, and PGE2 is increased in bone marrow mesenchymal stem cells in response to IL-1 exposure [39]. The expression of IL-1β and TNF-α is driven by NF-κB, and in turn, they are also potent activators of the NF-κB pathway. The content of cytokines, especially chemokines, in macrophage-derived exosomes increases after LPS stimulation [40]. Similar experimental studies found elevated levels of chemokine (CC motif) ligand 3 (CCL3), also called MIP1α (macrophage inflammatory protein-1α), in exosomes secreted by macrophages after LPS treatment [41]. CCL3 acts as a chemotactic factor responsible for the recruitment of monocytes/macrophages at sites of inflammation. In prosthetic aseptic loosening, polymethylacrylate particles stimulate RAW 264.7 cell-induced MSC chemotaxis due to CCL3 as it can be blocked by CCL3 inhibitors [42]. In addition, exosomes isolated in monocytes were found to transport arachidonic acid, a precursor of the inflammatory factor PGE2, into fibroblast-like synoviocytes of patients with rheumatoid arthritis [43]. PGE2 is a paracrine factor released from mesenchymal stem cells with powerful immunomodulatory functions and upregulates IL-10 secreted by macrophages and stimulates M2 macrophage polarization to accelerate the recovery of damaged tissues [44]. However, PGE2 also promotes RANKL expression in periprosthetic fibroblasts [45].

Functionally, exosomes containing inflammatory factors resemble inflammatory cells, modulate the expression of inflammatory responses, and have an essential role in the regulation of inflammatory bone disease. However, a complete spectrum of exosome-associated inflammatory cytokines is still not available, probably due to the inability of the technology to completely distinguish conventional cytokines from those in exosomes. Therefore, the changes in the content of and the intensity of action of these inflammatory factors in exosomes of skeletal aseptic inflammation have not been well studied.

3.3. Exosomal Delivery of RNA Promotes Macrophage Polarization. Exosomes can transport inflammatory factors and control the expression of inflammatory factors to promote the inflammatory response. Exosomes from IL-1β-treated synovial fibroblasts could induce osteoarthritis-related gene expression changes in articular chondrocytes, including upregulation of MMP-3, MMP-13, IL-6, and VEGF [46]. Another prominent example is that exosomes secreted by TNF-α-treated monocytes and T cells can directly stimulate the secretion of inflammatory mediators IL-6 and IL-8 by fibroblast-like synoviocytes [43]. Exosomes derived from SF cells from patients with end-stage osteoarthritis promote the macrophage expression of a range of proinflammatory factors, such as IL-1β, IL-6, chemokines, MMP-7, and MMP-12 [47].

Several studies have further explored the mechanisms through which exosomes promote inflammatory responses. The expression levels of exosomes containing lncRNA Hotair were significantly elevated in rheumatoid arthritis sera, leading to the migration of activated macrophages [48]. This phenomenon may be attributed to lncRNA Hotair that regulates the activation of NF-κB and the expression of its target genes (IL-6 and iNOS) by promoting IκBα degradation [49]. In rheumatoid arthritis, miR-106b is highly expressed in fibroblast-derived exosomes [50]. miR-106b promotes macrophage polarization and increases osteoclast formation by activating phosphatase and tensin homolog/-phosphatidylinositol 3-kinase/serine/threonine-protein kinase (PTEN/PI3K/AKT) and NF-κB signaling pathways [51]. miR-106b promotes the phosphorylation of IκB-α and p65, thereby facilitating the activation of NF-κB signaling in macrophages.

As such, these inflammation-induced exosomes affect macrophage activation via NF-κB signaling. These may also help to reveal the mechanism of TNF-α- and IL-1-induced NF-κB activation. NF-κB, in turn, is responsible for the transcription of many genes for proinflammatory cytokines and chemokines. These exosomes, which have a proinflammatory response, form positive feedback with inflammatory factors and promote the development and progression of inflammatory bone disease.

3.4. Exosomes Prevent Overactivation of the Inflammatory Response. Macrophage-derived exosomes not only promote the development of aseptic inflammation but also play a role in preventing the overactivation of the immune response. LPS-stimulated macrophages secrete exosomes carrying higher levels of three miRNAs (miRNA-21-3p, miRNA-146a, and miRNA-146b) than those of unactivated macrophages. These three miRNAs can inhibit the release of inflammatory factors from macrophages by suppressing NF-κB expression and Toll-like receptor activation [52, 53]. In addition, further studies revealed that miR-21, induced by NF-κB, can act as an inflammatory suppressor involved in the regulation of protective cytokines IL-4 and IL-10 [54, 55]. It may partially explain why IL-10 is elevated in patients with aseptic loosening of the prosthesis. Another study reported that macrophage-derived exosomes have high levels of miR-223 [56]. miR-223 suppresses inflammatory responses by targeting IKKα and inhibiting nonclassical NF-κB signaling during macrophage differentiation [57, 58]. However, miR-223 can induce differentiation of monocytes to macrophages by regulating inositol phosphatase, which is essential for monocyte survival [56].

Mesenchymal stem cell-derived exosomes are the most vital force that prevents overactivation of the inflammatory response in aseptic inflammation of the bone. The anti-inflammatory effects of MSC-derived exosomes have been described in several scenarios. Several studies have shown that MSC-derived exosomes can attenuate macrophage polarization and recruitment. This finding is exemplified in work undertaken by Shen et al. [59]. They found that MSC-derived exosomes express CC motif chemokine receptor 2, which plays a crucial role in preventing macrophage accumulation and tissue damage by inhibiting its activity through binding to the proinflammatory chemokine CCL2 as a decoy receptor. CCL2, also called MCP-1, is the most critical chemokine that regulates the migration of monocytes and macrophages. Macrophages and monocytes migrate toward a slope of CCL2 via its receptor CCR2. Titanium

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particles lead to increased CCL2 secretion [60]. Cosenza et al. demonstrated for the first time that MSC-derived exosomes play an immunomodulatory role in inflammatory arthritis [61]. In addition, various disease models have illustrated the effect of MSC-derived exosomes on the M1/M2 polarization of macrophages. Gingival tissue-derived MSCs showed a rapid increase in exosome secretion after TNF-α treatment, which also enhanced CD73 exosome expression and promoted the polarization of M2 macrophages [62]. Several studies have further investigated the regulatory mechanisms through which MSC-derived exosomes affect macrophage polarization. MSC-derived miR-150-5p exosomes reduced migration and invasion of fibroblast-like synovial cells in patients with rheumatoid arthritis by targeting MMP-14 and VEGF [63]. In another study, Xu et al. found that exosomes derived from bone marrow MSCs pretreated with LPS reduced the phosphorylation level of IκB, thus inhibiting the LPS-dependent NF-κB signaling pathway [64]. However, they did not indicate the exosome component that caused this result. These findings may provide new ideas for the treatment of inflammatory bone diseases.

Taken together, macrophages can secrete several exosomes containing unique microRNAs that are involved in preventing the overactivation of the inflammatory response. In addition, MSC-derived exosomes may have similar effects to MSCs and are major anti-inflammatory exosomes. They can induce macrophage shift from the M1 phenotype to the M2 phenotype, inhibit proinflammatory cytokines, release anti-inflammatory cytokines, inhibit the progression of inflammatory bone diseases, and attenuate periprosthetic osteolysis. Although the inflammatory response is essential in resisting various external stresses, it is also a double-edged sword. During inflammation, exosomes released by various cells are simultaneously inhibiting the course of the inflammatory response, which may be a self-protective effect of the organism. Nevertheless, this coordination of anti-inflammatory effects does not entirely stop the progression of inflammatory bone disease. The exosomes contain goods with both proinflammatory and anti-inflammatory effects to buffer the inflammatory response of the receptor cells to an optimal magnitude of response. This simultaneous proinflammatory and anti-inflammatory mechanism results in a weak inflammatory state, which allows the disease to last for years or even decades.

4. Exosomes and Bone Metabolism

4.1. Exosomes and Bone Resorption. Osteolysis due to increased osteoclast activity is the primary pathological process in the development of inflammatory bone disease to a specific stage. The osteoclast, whose specific marker is the expression of tartrate-resistant acid phosphatase (TRAP), is the only cell type that participates in the destruction and resorption of bone tissue in living organisms. Osteoclasts are differentiated from hematopoietic stem cell-derived monocytes and macrophage lineage progenitors (progenitors of osteoclasts). Osteoblasts secrete the macrophage colony-stimulating factor (M-CSF) and act on osteoclast progenitors to promote their survival, while the receptor activator of nuclear factor-kappaB ligand (RANKL) is secreted to promote osteoclast precursor cell differentiation [65]. When RANKL binds to the receptor activator of nuclear factor-kappaB (RANK) expressed in the osteoblast precursor cells, ligand proteins, such as tumor necrosis factor receptor-associated factor-6 (TRAF6), bind to the intracellular domain of RANK. Then, kinases, such as NF-κB, extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38 are activated [66]. Eventually, osteoclast precursor cells differentiate into osteoblasts by expressing c-Fos and nuclear factor of activated T cells (NFATc1), transcription factors of specific genes, such as tartrate-resistant acid phosphatase (TRAP), and cathepsin K, for osteoclast differentiation [67, 68]. As in inflammation, exosomes are involved in several processes, such as promoting osteolysis and inhibition of osteolysis (Figure 3).

4.1.1. Exosomes Directly Promote Differentiation of Osteoclasts. Osteoclasts can take up exosomes by phagocytosis and are affected by the various cargoes carried by exosomes [69]. RANKL, a growth factor essential for osteoclast differentiation, was identified in 2015 as expressed in exosomes secreted by osteoblasts and stromal cells [70]. Isolation of RANKL-containing exosomes from mouse cranial osteoblasts was shown to stimulate the differentiation of monocytes/macrophages into osteoclasts [71]. Recent in vivo experiments have demonstrated that RANKL-containing exosomes released from osteoblasts can target osteoclasts and stimulate osteoclast formation [72]. The targeted delivery of RANKL-containing exosomes to osteoclasts may be related to the interaction of RANKL on the surface of the exosome membrane with RANK on the surface of the osteoclast precursor membrane [70]. Studies have shown that after parathyroid hormone treatment, osteoblasts secrete more RANKL-containing exosomes, causing an increase in osteoclast differentiation [73]. In another study, RANKL levels in synovial exosomes were significantly higher in patients with rheumatoid joints [74]. Exosomes likely promote osteoclast differentiation directly through RANKL transport. Nevertheless, now, RANKL levels in exosomes are not well measured in other sterile bone inflammatory conditions, such as aseptic loosening.

In addition, Li et al. found that miR-214 expression in osteoblasts was positively correlated with miR-214 levels in serum exosomes and that patients with osteoporotic fractures had higher miR-214 levels than normal subjects [75]. Further studies showed that miR-214 targeted to block PTEN expression and activated the PI3K/Akt signaling pathway, thereby enhancing osteoclast formation [76]. Exosomes containing miR-106b can also promote osteoclastogenesis by regulating the PTEN/PI3K/Akt pathway [51]. PTEN is a repressor gene of Akt and inhibits Akt activation by dephosphorylating PIP3 to antagonize the activity of PI3K. The activated PI3K/Akt pathway inhibits glycogen synthase kinase 3β (GSK-3β) through phosphorylation, and this GSK-3β inhibition leads to nuclear localization of NFATc1, resulting in enhanced osteoclastogenesis [77].

Exosomes can regulate osteoclast activity by promoting the expression of inflammatory factors and by direct

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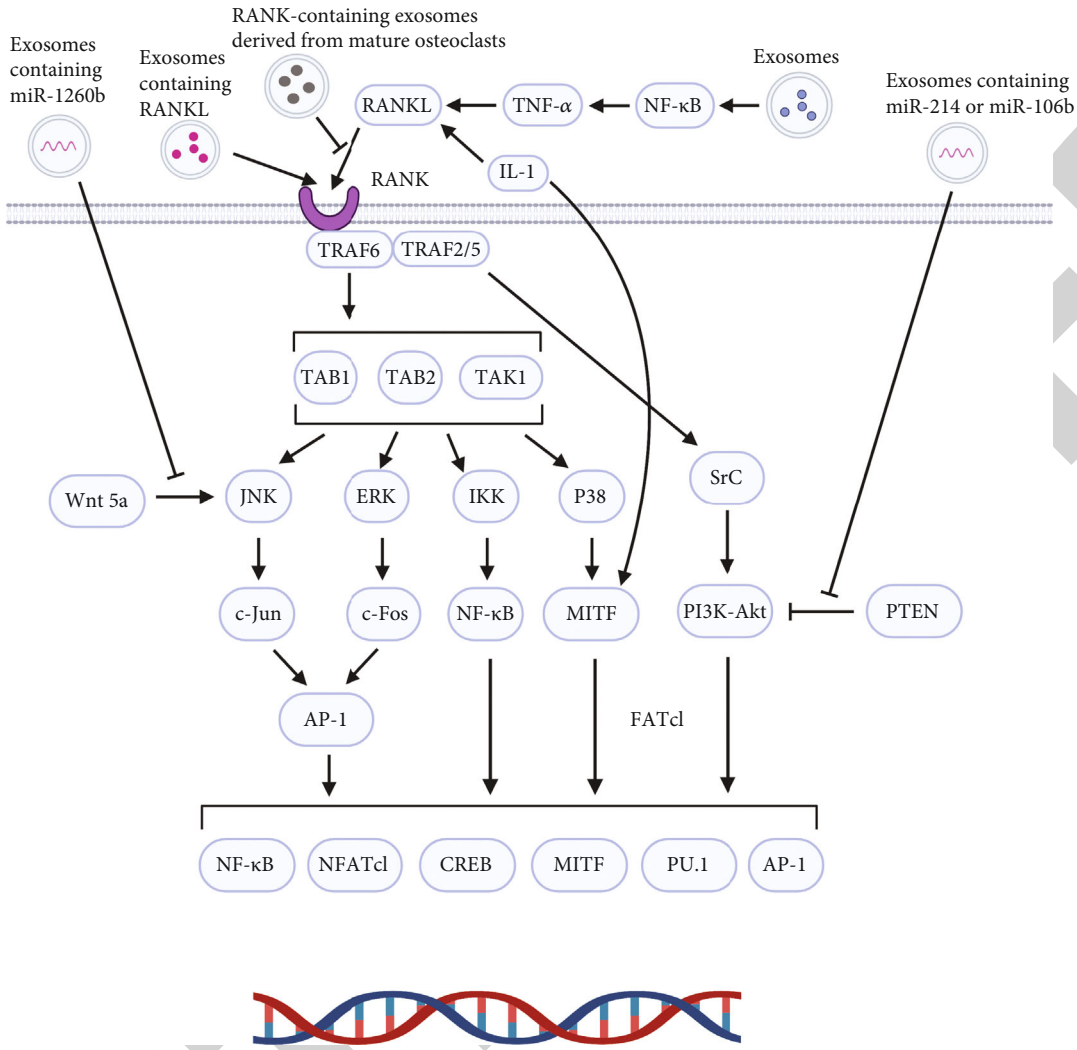

Figure 3: Effect of exosomes on osteoclasts. The stimulation of RANK by RANKL initiates a long series of downstream signaling and gene expression cascades. Exosomes with proinflammatory effects increase the expression of inflammatory cytokines TNF-α and IL-1, which promote the expression of RANKL and enhance the sensitivity of osteoclasts to RANKL. IL-1 can also promote osteoclast maturation directly through MITF. Exosomes containing miR-214 or miR-106b promote osteoblast maturation by blocking the inhibitory effect of PTEN on the PI3K-Akt signaling pathway. RANKL-containing exosomes bind directly to RANK on the surface of osteoclasts and activate the RANK signaling pathway. By contrast, miR-1260b-containing exosomes inhibit the activation of Jun-N terminal kinase (JNK) by Wnt5a to suppress RANKL signaling. Mature osteoblast-derived RANK-containing exosomes inhibit RANKL signaling by competitively binding to RANKL.

activation of osteoclasts via transported cargo. Multiple mechanisms act together to trigger osteolysis in sterile bone inflammation.

4.1.2. Exosomes Indirectly Promote Osteoclast Differentiation through Inflammatory Factors. Some specific exosomes derived from inflammatory bone diseases promote the expression of a range of inflammatory factors, including TNF-α, IL-1, and VEGF, which increase osteoclast activity [78, 79]. TNF-α promotes periprosthetic bone resorption by enhancing RANKL and M-CSF expression in osteoblasts, osteocytes, and stromal cells and enhancing the sensitivity of osteoclast precursors to RANKL [80]. This mechanism of promoting RANKL-induced osteoclast formation may be accomplished by inducing the expression of Blimp1, a transcriptional repressor that plays a critical role in the differentiation and function of a variety of cells, including osteoclasts [81]. However, in the absence of RANKL and osteoblast/stromal cells, TNF-α stimulates osteoclast differentiation in the presence of M-CSF [82]. Although transient exposure of bone marrow MSCs to TNF-α increases osteogenesis in vitro, continued stimulation with TNF-α leads to osteoclast activation and impaired osteoblast function. IL-1, another inflammatory factor promoted by expression in sterile bone inflammatory exosomes, enhances RANKL expression in osteoblasts and stromal cells and may directly stimulate the differentiation of osteoclast precursors through an alternate pathway (RANKL independent). This RANKL-independent osteoclast induction may be related to IL-1α-induced expression of microphthalmia transcription factor

(MITF) in macrophages [83]. VEGF is also carried by exosomes as a cargo [84]. VEGF acts as an osteolytic agent in a paracrine manner in inflammatory bone diseases. It also plays a role in the chemotaxis and proliferation of osteoclast precursors, inducing osteoclastogenesis [85].

4.1.3. Exosomes Affect the Degradation of the Extracellular Matrix. Exosomes derived from monocytes and T cells stimulated by inflammatory factors, such as TNF-α, induce high production of MMP-1, MMP-3, MMP-9, and MMP-13 by rheumatoid arthritis fibroblasts [86]. Matrix metalloproteinases (MMPs) are a family of 23 structurally related proteolytic enzymes, which can degrade almost all components of the extracellular matrix [87]. MMP-9 promotes bone resorption by degrading extracellular matrix macromolecules around and on the surface of bone trabeculae and mediates osteoclast adhesion and migration to resorption sites [88]. MMP-13, which breaks down proteoglycans in the extracellular matrix, such as proteoglycans and collagen, is thought to be the main protease responsible for cartilage destruction in rheumatoid arthritis [78]. Several studies demonstrated that many MMPs might lead to prosthetic loosening and osteolysis through pathological extracellular matrix degradation and periprosthetic connective tissue/bone remodeling [89–92]. Periprosthetic fibroblast-like cells express MMP-13 in response to induction of wear particles, and the increase in matrix metalloproteinases destroys the periprosthetic tissue [45]. Blocking TNF-α and IL-1β receptors does not attenuate the damaging effects of exosomes on the extracellular matrix, suggesting that the mechanism of increased matrix metalloproteinase production caused by exosomes is independent of the TNF-α-induced inflammatory response. Exosomes secreted by fibrous synovial cells from rheumatoid patients carry high levels of ADAMTS-5, and exosomes isolated from endothelial cells contain MMP-2, MMP-9, and MMP-14 [78]. This finding suggests that exosomes from patients with aseptic bone inflammation can break down bone tissue directly. Exosomes directly mediate the destruction of the bone matrix through matrix metalloproteinases, which is an essential mechanism for the occurrence of osteolysis.

4.1.4. Exosomes Prevent Excessive Osteolysis. Exosomes from mature osteoclasts can inhibit osteoclast formation through a paracrine mechanism [93, 94]. Exosomes from plasma of rheumatoid arthritis (RA) patients are similar to those in normal human plasma and significantly inhibit osteoclast production [95]. This inhibitory effect may be due to the role of the receptor RANK in mature osteoclast-derived exosomes as a decoy receptor that competitively binds RANKL similar to OPG. Further analysis showed that RANK levels in plasma exosomes are higher in RA patients than in normal subjects. This finding may be due to the increased production of mature osteoblasts. As such, osteoclast-derived exosomes may have a role in preventing excessive osteolysis persisting in inflammatory bone diseases. This process may involve a negative feedback regulation by the body to maintain the balance of bone metabolism and prevent excessive osteolysis. In another study, TNF-α pretreated MSCs secreted miR-1260b-containing exosomes in large quantities [62]. Exosomes containing miR-1260b target and block the Wnt5a-mediated RANKL pathway and inhibit osteoclast activity. MSC-derived exosomes have inhibitory effects not only on inflammation but also on osteoclast maturation. MSC-derived exosomes might be an excellent therapeutic direction for inflammatory bone diseases.

4.2. Exosomes and Bone Formation. Bone metabolism is strictly regulated by a balance of bone formation by osteoblasts and bone resorption by osteoclasts. Bone loss may be associated with increased osteoclast activity, reduced osteoblast activity, or a combination of both. Increased osteoclastic activity and decreased osteogenic activity play a role in inflammatory bone diseases. Osteoblasts, derived by direct differentiation of bone marrow mesenchymal stem cells, are responsible for bone formation in bone remodeling in vivo. Runt-related transcription factor-2 (Runx2) and osterix (OSX) are specific transcription factors for all osteoblast proliferation and differentiation stages, facilitating skeletal formation by transactivating bone matrix protein genes, including collagen type I, osteocalcin, and osteopontin [96, 97]. Exosomes in aseptic bone inflammation can affect osteoblast differentiation and activity in multiple ways by acting directly or indirectly on the Wnt signaling pathway or the expression of the transcription factors Runx2 and OSX (Figure 4).

4.2.1. Exosomes Directly Affect the Activity of Osteoblasts. In aseptic inflammation of the bone, exosomes can directly influence the differentiation of osteoblasts, in addition to influencing osteoblast formation through inflammatory factors. Macrophage-derived exosomes are the leading force in inhibiting osteoblast differentiation in inflammatory bone diseases. miR-155 is enriched in M1 macrophage-derived exosomes [98]. miR-155 significantly decreases the expression of BMP2, BMP9, and Runx2, thus inhibiting osteogenic differentiation of MSC. miR-23a-5p-containing exosomes induced by RANKL are highly expressed in RAW 264.7 cells [99]. miR-23a-5p-containing exosomes effectively inhibit Runx2 and promote Yes-associated protein-1- (YAP1-) mediated MT1DP by suppressing osteoblast differentiation. Exosomes produced by RAW 264.7 cells after titanium particle treatment inhibit the differentiation of MC3T3-E1 cells [100]. The analysis of differentially expressed lncRNAs in exosomes produced by RAW 264.7 cells revealed that lncRNA NONMMUT000375.2 and lncRNA NONMMUT071578.2 might play an essential role in inhibiting osteoblast differentiation. However, no relevant experiments have explored the mechanism of action of these two lncRNAs involved in regulating osteoblast differentiation.

In addition, exosomes in the serum of patients with aseptic bone inflammation may also have remarkable effects on the differentiation of osteoblasts. In serum samples from patients with osteoporosis and ovarian denuded mice, the levels of exosomal miRNA were significantly higher than normal reference values [101]. Exosomes derived from osteoclasts containing miRNA-214 are delivered to osteoblasts through the ephrinA2-EphA2 signaling pathway and

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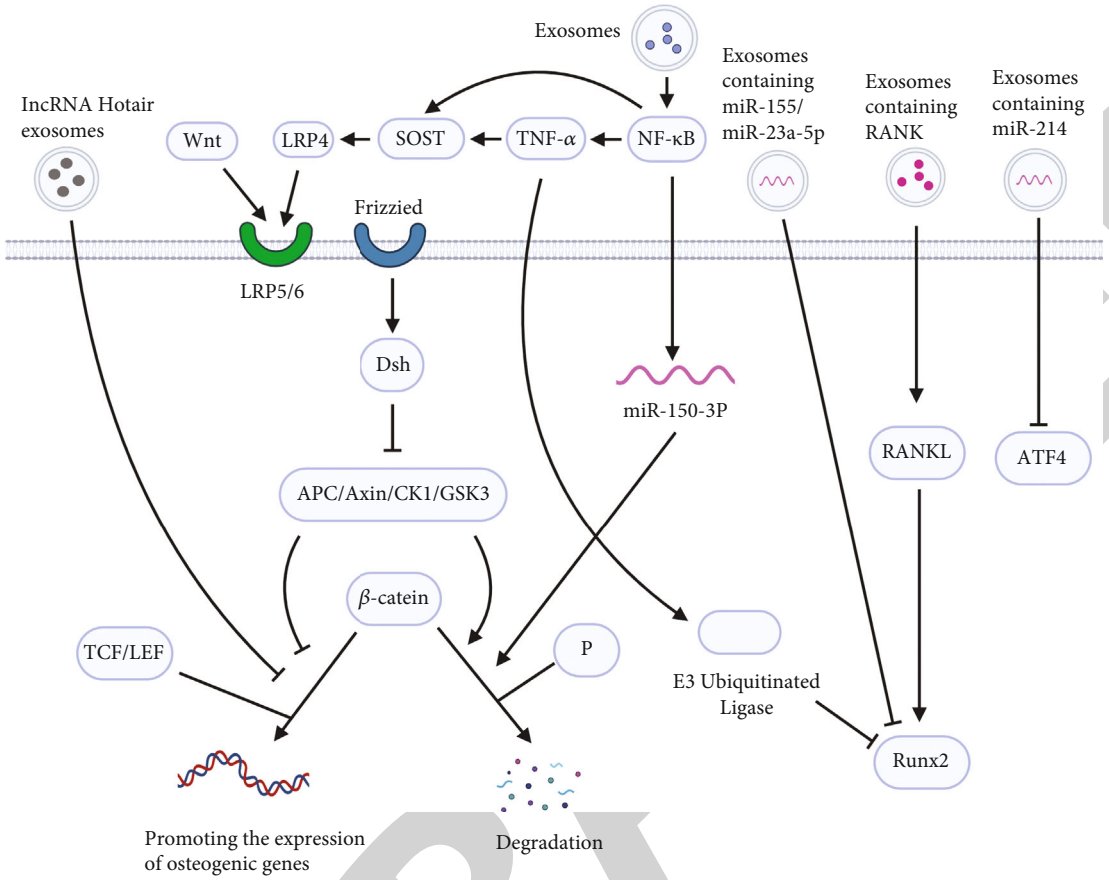

Figure 4: Process through which exosomes affect osteoblasts. In the canonical pathway, Wnt signaling promotes β-catenin translocation to the nucleus, interacts with TCF/LEF, and stimulates the expression of downstream genes. Macrophage-derived exosome-induced inflammatory cytokines promote SOST expression, and the molecular chaperone LRP4 presents sclerostin to the Wnt coreceptors LRP5/6, thereby promoting inhibition of Wnt/β-catenin signaling by sclerostin. NF-κB inhibits the degradation of β-catenin via miR-150-3p. TNF-α represses the expression of transcription factor Runx2 via E3 ubiquitination ligase. lncRNA Hotair inhibits the nuclear localization of TCF/LEF and β-catenin, thereby suppressing Wnt signaling. miR-155 and miR-23a-5p in exosomes directly repress Runx2 expression, while miR-214 represses activating transcription factor-4 (ATF4) expression. RANK signaling in exosomes triggers RANKL reverse signaling and promotes Runx2 expression.

negatively regulate ATF4, thereby inhibiting osteogenic activity [102]. ATF4 is a crucial regulator of bone formation and determines the initiation and terminal differentiation of osteoblasts by transactivating the osteocalcin (OCN) gene and promoting the expression of other osteogenic genes, such as bone sialoprotein (BSP) and OSX [103]. In addition, lncRNA Hotair is classified as an essential regulator and is highly expressed in serum, which makes it a potential factor for the diagnosis of rheumatoid arthritis by serum assays [18]. Recent studies suggest that the mechanism of action of lncRNA Hotair may be related to the inhibition of osteogenic differentiation of MSCs by downregulating the expression of proteins related to the Wnt/β-catenin pathway [104].

4.2.2. Exosomes Indirectly Affect Osteoblasts. The interaction of exosomes with inflammatory factors is a force to be reckoned with in inflammatory bone diseases. Exosomes promote the expression of inflammatory factors, such as TNF-α in inflammatory bone diseases, through various mechanisms, and these inflammatory factors inhibit osteoblast differentiation through various ways. TNF-α is one of the most potent inhibitors of osteogenic differentiation [105]. TNF-α independently inhibits the expression of procollagen α1 mRNA, resulting in reduced type I collagen synthesis that is a significant component of the mineralized bone matrix [106]. TNF-α can also induce the production of Dickkopf-1 (DDK1) and sclerotin (SOST), inhibitors of Wnt signaling, to inhibit osteoblast differentiation [107]. In addition, TNF-α can upregulate E3 ubiquitination ligase, which mediates the ubiquitination and degradation of Runx2, a key transcription factor for osteoblast differentiation [108–110]. Activation of NF-κB also inhibits osteoblast production. The activation of NF-κB upregulates miRNA-150-3p, inhibiting osteogenesis of mesenchymal stem cells by encouraging β-catenin degradation [111]. Besides, NF-κB has a direct role in inhibiting BMP and Wnt signaling and negatively regulates bone mass through sclerostin [112]. Exosomes that activate inflammatory cytokines ultimately promote inflammatory bone diseases by promoting osteoclast formation and inhibiting osteoblast formation.

4.2.3. Exosomes Prevent Excessive Reduction in Osteoblast Activity. While exosomes inhibit osteoblast activity in inflammatory bone diseases, exosomes that promote osteoblast activity are also produced due to the self-protection of the organism. One well-known example is that osteoclasts produce exosomes that promote osteoblast formation. Osteoclasts release RANK-containing exosomes that bind to RANKL on osteoblasts and stimulate RANKL to reverse signaling to promote osteoblast differentiation and bone formation via Runx2 [113]. Marton et al. showed higher levels of RANK in exosomes from patients with rheumatoid arthritis than in normal individuals [95]. However, whether this mechanism is self-protective and promotes osteoblast differentiation in aseptic inflammation of bone is unknown. Whether the reverse signaling of RANK-activated osteoblasts in exosomes plays a role in various bone metabolic diseases should be further investigated. Another important mechanism is that TNF, whose expression is facilitated by exosomes, can induce NFATc1 and promote bone formation [114]. Many experiments have observed a paradoxical role of NFATc1 in bone formation. It is unclear whether the dysregulation of bone formation in inflammatory bone disease is a coupled effect of increased bone loss leading to the increased bone formation or whether inflammation directly induces bone formation. Besides, exosomes containing miR-29b are highly expressed in TNF-α-treated RAW 264.7 cells. miR-29b may be associated with the regulation of osteogenic and osteolytic differentiation in aseptic inflammation of the bone [115]. Recent studies found that first miR-29b can promote proliferation and migration of rat bone marrow MSCs through PI3K/AKT and TGF-β/Smad signaling pathways [116]. These protective effects seen in inflammatory bone diseases may be related to the osteoblast-osteoclast dynamic balance, but further research is needed to elucidate the exact mechanisms.

5. Exosomes, Inflammation, and Bone Metabolism

Inflammation has the physiological purpose of restoring tissue homeostasis. However, uncontrolled or unresolved inflammation can lead to tissue damage, resulting in a variety of diseases characterized by a chronic inflammatory state [117]. Indeed, systemic inflammatory pathways are an essential component in the pathogenesis of inflammatory bone diseases. Inflammatory changes in the bone microenvironment can lead to excessive bone loss and altered bone formation. Inflammatory processes are associated with altered systemic bone remodeling, increased bone resorption, and impaired bone formation, and inflammatory mediators affect the differentiation and activity of osteoclasts and osteoblasts. Proinflammatory cytokines can enhance osteoclastogenesis and osteoclast activity. A decrease in bone-forming cell activity is also observed during the inflammatory process. The use of anti-inflammatory drugs (including glucocorticoids) can protect bones from inflammation-induced bone fragility. The inflammatory response occurs as an etiology of the disruption of the balance of bone metabolism. Exosomes appear to play a critical role in multiple signaling cascade responses in the inflammatory process because of their ability to carry inflammatory regulators, such as miRNAs and proteins, that can act on proximal and distant target tissues. Exosomes indirectly affect the activity of osteoblasts and osteoclasts by modulating the inflammatory response, thereby disrupting bone metabolic homeostasis. The loss of bone metabolic balance causes a range of symptoms, including inflammatory osteolysis and osteoporosis.

Homeostasis in bone metabolism is determined by the delicate balance between bone resorption by osteoclasts and bone formation by osteoblasts. This balance can be maintained between osteoblasts and osteoclasts through a variety of signaling pathways, such as RANKL/RANK, Ephrin/Eph, Wnt, complement, and TGF coupled together. Both osteoblast- and osteoclast-derived exosomes are involved in the coupling effect [118]. In an inflammatory environment, altered exosome expression can affect the coupling between osteoblasts and osteoclasts, thereby affecting the homeostasis of bone metabolism. Osteoclast-derived coupling factor, an exosome containing miR-214-3p, is altered in inflammatory bone disease, thereby affecting normal bone metabolism. As a coupling factor secreted by osteoblasts, miR-503-3p-containing exosomes can inhibit osteoclast differentiation. miR-677-3p, miR-680, miR-3084-3p, and miR-5000 are highly expressed in exosomes derived from mineralized osteoblasts. Let-7 is found in exosomes derived from osteoblast precursors and mature osteoblasts and can enhance osteogenesis by regulating the high mobility groups AT-hook 2 (HMGA2) and Axin 2. Osteoblast-derived exosomes containing miR-30d-5p, miR-140-3p of miR-133b-3p, miR-335-3p, miR-378b, and miR-677-3p also act as coupling factors that regulate osteoblast differentiation by autocrine means [119]. However, the role of exosomes and their level changes in bone homeostasis as osteoblast-osteoclast coupling factors in inflammatory bone diseases have not been well studied.

Overall, in inflammatory bone diseases, oxidative stressors can induce specific exosomal changes that aid in the inflammatory response. The inflammatory response subsequently affects the activity of osteoclasts and osteoblasts through the secretion of inflammatory factors or exosomes. It seems that the creation of an inflammatory microenvironment results in corresponding changes in exosomes, which are coupling factors in normal bone homeostasis, causing disturbances in bone metabolism. However, it is unclear whether these changes in the exosomes of osteoblast-osteoclast communication are caused by inflammation per se or by feedback changes following disturbances in bone metabolism. Finally, disturbances in bone metabolism in response to inflammation lead to increased bone loss and ultimately to inflammatory bone disease.

6. Clinical Application of Exosomes in Inflammatory Bone Diseases

One potential application of exosomes in inflammatory bone diseases is their use as a diagnostic and prognostic biomarker. The cargo in exosomes is highly dependent on the

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state of the releasing cell and its microenvironment. Thus, any pathological changes in the tissue microenvironment are reflected in the cargo content of the exosomes they release. Healthy subjects and patients with aseptic bone inflammation release exosomes containing varying concentrations of proteins, RNA, and other components into the circulation, which can be measured as biomarkers. Exosomes are widely available in a variety of body fluids and can be readily accessed. The exosomes encase the cargo and maintain its stability outside the cell. Techniques, such as reverse transcription-quantitative PCR, allow sensitive and specific detection of biomarkers, such as miRNAs in plasma and other body fluids. However, the technique of exosome purification still has many shortcomings. For example, as the most widely used method for the isolation of exosomes, differential centrifugation suffers from many disadvantages, such as coseparation of nonexosomal impurities, low reproducibility, low RNA yield, potential damage to exosomes, and low sample utilization [120]. Several studies have collectively shown differences in the fractions of exosomes extracted by different extraction methods [121–123]. The actual exosome content must be carefully differentiated by proper separation methods to prevent errors due to the cargo from other body fluid sources. In the future, if exosomes are used in clinical diagnostics, the method of exosome isolation must be improved to ensure reliable and reproducible results. Although few of these exosomal biomarkers have been documented to date, these exosomal components have considerable potential as diagnostic tools.

Exosomes of different origins and containing different components may exhibit inflammatory inhibition or osteolysis inhibition, making them potential tools for the treatment of inflammatory bone diseases. As natural endogenous nanovesicles, exosomes have various advantages, such as low immunogenicity, nontoxicity, and higher stability than other synthetic nanoparticles. The use of exosomes as an alternative to cellular therapies may be more straightforward, safer, and less costly, avoiding many of the problems associated with parental cellular drug delivery. In addition, exosomes can cross a variety of tissue barriers, enhancing the therapeutic effect of the adulterated molecules on target cells. Exosomes are used for three primary purposes in the treatment of inflammatory bone diseases: to modulate the immune response, to inhibit bone resorption and initiate bone repair, or to act as a carrier for therapeutic agents.

A massive example of modulating the immune response is the use of MSC-derived exosomes to suppress the inflammatory response. This method has been attempted in several experiments with promising results [124–128]. MSC-derived exosomes can ameliorate IL-1β-induced inflammatory effects and reduce apoptosis and matrix degradation during repair, as well as influence the conversion of macrophages to the M2 phenotype and participate in anti-inflammatory and regenerative responses. MSC exosomes may exert anti-inflammatory effects through specific miRNAs (miRNA-135b, miRNA-140-5p, miRNA21, miRNA-146a, and miRNA-181c), reversing the pathological inflammatory state without causing further apparent toxicity [129]. In a rat model, miRNA-135b in MSC-derived exosomes could promote cartilage repair by regulating TGF-β. Overexpression of miRNA-140-5p by exosomes derived from human synovial mesenchymal stem cells promoted cartilage tissue regeneration and blocked the side effects of OA [130]. The maturation mechanisms of miRNAs may vary depending on the cell type and cellular microenvironment. Mature MSC-derived exosomes contain more mature miRNAs than immature MSC-derived exosomes, and these miRNAs can promote chondrocyte proliferation, reduce apoptosis, and regulate immune responses [131]. Macrophage-derived exosomes may also contribute to the control of inflammation [132, 133]. The mechanisms through which these exosomes control aseptic inflammation should be further investigated. Further in vivo and clinical trials are needed to confirm its efficacy due to differences in vitro and in vivo and between organisms.

Osteoclast differentiation and function are responsible for inflammatory bone disease, accelerated by the control of cytokines produced in the inflammatory environment. The promotion of bone repair is another new target for the use of exosomes in the treatment of inflammatory bone diseases. For example, RANK-rich exosomes that inhibit osteoclast formation and promote osteoblast activity through reverse signaling can be used. However, better therapeutic outcomes may be achieved when treatment strategies that promote bone repair are used in conjunction with those that inhibit inflammation. Of course, exosomes also have a very high potential for drug delivery systems. Of particular interest is the macrophage-derived exosome expressing CD47, a surface signaling molecule used to evade immune surveillance. CD47 on the exosome membrane prevents endocytosis of exosomes by monocytes and macrophages, while CD9 and CD81 on the exosome surface promote the phagocytosis of exosomes. Macrophage-derived exosomes are therefore very attractive as drug delivery vehicles for the treatment of inflammatory bone diseases. Using these phagocytic properties, we can deliver drugs to target cells more efficiently. However, no studies have been conducted to prove whether such exosomes can be used to deliver anti-inflammatory drugs to reduce inflammatory diseases. Moreover, despite the efforts that have been made in these directions, substantial obstacles remain in obtaining exosomes with the desired properties.

7. Summary and Prospect

Exosomes have multiple functions similar to those of mother cells and have received increasing attention due to their natural portability and unique effects on target cells. With further research, we find that exosomes are closely associated with the pathological process of aseptic bone inflammation. Exosomes affect macrophage polarization and regulate the inflammatory response mainly through the NF-κB signaling pathway. Alterations in inflammatory cytokines and exosome secretion caused by inflammation further affect bone metabolic homeostasis. These signals promote osteoclast differentiation via the RANKL pathway and inhibit the osteoblast activity via the Wnt pathway and the transcription

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factor Runx2, causing inflammatory osteolysis and promoting the development and progression of inflammatory bone disease. However, the body also produces exosomes that have a protective effect against the disease process. Exosomes have shown new pathogenesis in inflammatory bone diseases and provide a new direction for diagnosis and treatment. The use of exosomes has a very high potential in regulating inflammation and promoting bone repair. Reprogrammed or redesigned exosomes for disease treatment are promising in the future. However, the low yield and limited function of exosomes produced by current conventional methods essentially limit their further clinical application. Thus, further research on the biological properties of exosomes is urgently needed to improve the yields or enable the better function of exosomes. Overall, exosomes play an essential role in inflammatory bone diseases and are a promising therapeutic target.

Abbreviations

TNF-α: Tumor necrosis factor-α
IL: Interleukin
PGE2: Prostaglandin E2
NF-κB: Nuclear factor-kappaB
HMGB1: High mobility group box one
ILV: Intraluminal vesicles
MVB: Multivesicular body
EV: Extracellular vesicle
TLR: Toll-like receptor
LPS: Lipopolysaccharide
IFN-γ: Interferon γ
IRAK: Interleukin-1 receptor-associated kinase
TRAF: TNF receptor-associated factor
IKK: IκB kinase complex
NIK: NF-κB-inducing kinase
TAK1: Transforming growth factor β-activated kinase-1
MAPK: Mitogen-activated protein kinase
AP-1: Activator protein-1
MSC: Mesenchymal stem cell
MCP-1: Monocyte chemoattractant protein-1
CCL: Chemokine ligand
MIP: Macrophage inflammatory protein
PMMA: Polymethylacrylate
RANKL: Receptor activator of nuclear factor-kappaB ligand
VEGF: Vascular endothelial growth factor
MMP: Matrix metalloproteinase
iNOS: Inducible nitric oxide synthase
PTEN: Phosphatase and tensin homolog
CCR: Chemokine receptor
TRAP: Tartrate-resistant acid phosphatase
ERK: Extracellular signal-regulated kinase
JNK: Jun N-terminal kinase
NFATc1: Nuclear factor of activated T cell, cytoplasmic 1
MITF: Microphthalmia transcription factor
Akt: Serine/threonine protein kinase
PI3K: Phosphatidylinositol 3-kinase
ATF: Activating transcription factor
RANK: Receptor activator of nuclear factor-kappaB
GSK-3β: Glycogen synthase kinase 3β
SOST: Sclerotin
DKK1: Dickkopf-1
Runx2: Runt-related transcription factor-2
YAP: Yes-associated protein
OCN: Osteocalcin
BSP: Bone sialoprotein
OSX: Osterix
HMGA2: High mobility groups AT-hook 2.

Disclosure

Yingkun Hu, Yi Wang, and Tianhong Chen are co-first authors.

Conflicts of Interest

The authors report no conflicts of interest.

Authors' Contributions

Yingkun Hu, Yi Wang, and Tianhong Chen wrote original draft; Jingfeng Li and Lin Cai revised the original draft; Zhuowen Hao took part in the revision of the original draft; and Jingfeng Li was responsible for supervision. Yingkun Hu, Yi Wang, and Tianhong Chen contributed equally to this work.

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Research Article

Microglia-Derived Exosomes Improve Spinal Cord Functional Recovery after Injury via Inhibiting Oxidative Stress and Promoting the Survival and Function of Endothelia Cells

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Traumatic spinal cord injury (SCI) is a devastating disease of the central nervous system with long-term disability and high mortality worldwide. Revascularization following SCI provides nutritional supports to rebuild and maintain the homeostasis of neuronal networks, and the subsequent promotion of angiogenesis is beneficial for functional recovery. Oxidative stress drastically produced following SCI has been contributed to endothelial dysfunction and the limited endogenous repair of microvasculature. Recently, exosomes, being regarded as potential therapeutic candidates for many kinds of diseases, have attracted great attentions due to its high bioavailability, safety, and stability. Microglia have been reported to exhibit proangiogenic function and guide the forming of vasculature during tissue repair. However, the specific role of microglia-derived exosomes (MG-Exos) played in SCI is still largely unknown. In the present study, we aimed to evaluate whether MG-Exos could protect spinal cord microvascular endothelial cells (SCMECs) against the toxic effects of oxidative stress, thus promote SCMECs' survival and function. We also investigated the protective effects of MG-Exos in the mouse model of SCI to verify their capability. Our results demonstrated that MG-Exo treatment significantly decreased the level of oxidative stress (ROS), as well as did the protein levels of NOX2 when bEnd.3 cells were exposed to H_2O_2-induced oxidative stress *in vitro* and *in vivo*. Functional assays showed that MG-Exos could improve the survival and the ability of tube formation and migration in H_2O_2-induced bEnd.3 *in vitro*. Moreover, MG-Exos exhibited the positive effects on vascular regeneration and cell proliferation, as well as functional recovery, in the mouse model of SCI. Mechanically, the keap1/Nrf2/HO-1 signaling pathway was also investigated in order to unveil its molecular mechanism, and the results showed that MG-Exos could increase the protein levels of Nrf2 and HO-1 via inhibiting the keap1; they also triggered the expression of its downstream antioxidative-related genes, such as *NQo1*, *Gclc*, *Cat*, and *Gsx1*. Our findings indicated that MG-Exos exerted an antioxidant effect and positively modulated vascular regeneration and neurological functional recovery post-SCI by activating keap1/Nrf2/HO-1 signaling.

1. Introduction

Traumatic spinal cord injury (SCI) is a devastating disease of the central nervous system, which may cause major motor, sensory and autonomic dysfunctions, and bring far-reaching adverse consequences for patients, families, and society [1]. Epidemiological data showed that the number of prevalent cases of SCI, which had been rising for years, was

approximately 27.04 million, and there were estimatedly 0.93 million new cases annually throughout the world [2].

Contusion injury, followed by hypoxia, ischemia-reperfusion injury (SCIRI), and microenvironment imbalance, can cause the production and release of large amounts of free oxygen radicals and reactive oxygen species (ROS) [3, 4]. These strong oxidants accumulate in the tissues leading to topical microvasculature disorder and thus the dysfunction of myelin sheaths of nerve cells [5–8]. Studies have shown that oxidative stress is the main pathophysiological events leading to secondary damage for neural tissue and the parenchyma of the spinal cord in the injury epicenter [9, 10].

Posttraumatic vascularization ensures tremendous trophic support to build up and maintain homeostasis of neuronal networks [11–13]. Regenerating axons have been shown to grow along blood vessels [12, 14, 15]. However, high level of oxidative stress and poor microenvironment limited the endogenous repair of microvasculature and the regeneration of axons following SCI [16, 17]. Thus, quickly balancing the oxidative stress of the spinal cord is an important way to improve angiogenesis, restore blood flow, and protect the function of spinal cord microvasculature and nerve cells.

Recently, extracellular vesicles (EVs), such as exosomes and small microvesicles, are recognized as novel tools of intercellular communication [18]. Depending on their parental cells, these EVs contain a variety of specific sets of substances, including proteins, lipids, metabolites, and nucleic acids [18, 19]. Previous evidences showed that exosomes derived from mesenchymal stem cells (MSCs) or endothelial progenitor cells (EPCs) or adipose-derived stem cells (ADSCs) can protect cells such as epidermal keratinocytes and endothelial cells against the toxic effects of high oxidative stress and promote their survival rates [20–22]. In central nervous system, the neighbouring spatial localization of microglia and microvascular endothelial cells suggested an inseparable role of microglia in vascular angiogenesis and vasculopathy [23]. Mounting evidences are suggesting that microglia exhibit the proangiogenic function through forming perivascular clusters and secreting multiple factors such as VEGF (vascular endothelial growth factor), basigin-2, and FGF2 (fibroblast growth factor 2), which makes them key regulators and guides of angiogenesis in ischemic stroke and traumatic brain injury (TBI) [23–27]. Deletion of microglia in mice was found to have reduced vascular branch points in retina [28]. In the recent, Xu et al. have reported that microglia-derived exosomes (MG-Exos) can reduce the avascular areas of retinal in retinopathy of prematurity [29]. However, proangiogenic properties of MG-Exo in SCI angiogenesis and its underlying mechanism have not yet been investigated.

In this study, we determined that MG-Exos can protect the endothelial cells of spinal cord microvascular from the toxic effects of high oxidative stress and promote the function along with the survival rate of the endothelial cells.

2. Methods

2.1. Isolation, Culture, and Identification of Microglia. The primary mouse microglia were isolated and maintained as Saura et al. described [30]. Briefly, ten 1-day-old Bl/C57 mice were sacrificed by cervical dislocation. Afterwards, the mice's brains were isolated, the meninges and vessels were carefully separated with micro tweezers under microscope. The tissue was minced with a 1 ml tip pipette until the media became milky. Then, the tissue was digested with 0.25% trypsin (Sigma-Aldrich, USA) for 15 min at 37°C. After centrifuged and discarded supernatant, cells were seeded to cell culture plates with Dulbecco's Modified Eagle's Medium-F12 nutrient mixture (DMEM-F12, Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco), sodium pyruvate (1×), and 0.08% (w/v) gentamicin sulfate (Sigma). After cultured 10–12 days, cells were digested with 0.25% trypsin diluted 1 : 4 in DMEM-F12 for 25 min at 37°C to purify microglia.

The expression of surface marker proteins (including F4/80 (Abcam, USA) and Iba-1 (Wako, Japan)) on microglia was detected by immunofluorescence. The expression of surface marker proteins (including CD45, F4/80, CD11b, and CD68) on microglia was detected by flow cytometry.

2.2. Culture of bEnd.3 Cell Line. The bEnd.3 cells (brain-derived endothelial cells.3), immortalized mouse brain endothelial cells, were obtained from Advance Research Centre of Central South University (Changsha, China). They were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% penicillin, and streptomycin (Gibco). The bEnd.3 cells were maintained in a humidified incubator at 37°C with 5% CO_2 and 95% air.

2.3. Isolation and Identification of Microglia Exosomes. Cells were incubated in the completed medium containing exosome-depleted FBS (Vivacell, China) for 48 h. Microglia exosomes were collected from the completed medium by differential centrifugation as described previously [31]. In brief, the completed medium was centrifuged at 800×g for 10 min and 3000×g for 30 min at 4°C. After centrifugation at 10,000×g for 1 h at 4°C, the supernatant was filtered using a 0.22 μm filter (Millipore, USA) to remove cells and debris. The exosomes were purified twice by centrifugation at 140,000×g for 3 h and then resuspended for further characterization.

A transmission electron microscope (TEM; FEI company, USA) was further used to identify the form of the exosomes. Nanoparticle tracking analysis (NTA; ZetaView, Germany) was used to measure exosomes' diameter and particle number. The exosome markers CD9, tumor susceptibility gene 101 (TSG101), and CD63 were detected by western blotting analysis.

2.4. Exosome Uptake Assay. MG-Exos were labeled with a red fluorescent lipophilic dye PKH67 (Sigma) to monitor the motion of the exosomes. MG-Exos were resuspended in sterile PBS and incubated with 5 μM PKH67 for 15 min. Then, the suspension containing labeled exosomes was washed twice at 140,000×g for 90 min and resuspended in sterile PBS and used for the uptake experiment.

The bEnd.3 cells were seeded on chambered culture slides in 24-well cell culture plates until reaching 80% confluence and then incubated in the completed medium

containing 200 μg/ml PKH67-labeled exosomes at 37°C for 12 h. Culture slides were fixed with 4% paraformaldehyde (PFA; Biosharp, China) for 20 min and then incubated with 5% BSA (Biofroxx, Germany) in PBS for 30 min to block nonspecific staining. Nuclei were stained with DAPI (GeneTex Inc., USA), and signals were analyzed with a fluorescence microscope (Zeiss Apotome 2, Zeiss, Germany).

2.5. CCK-8 Assay. 100 μl cell suspension (5×10^3 cells) was seeded into each well of 96-well culture plates and treated with exosomes (200 μg/ml) or PBS. Four wells of replicates were set up in each group, and a group without cells served as the blank. After incubated in completed medium with 500 μM H_2O_2 for 2, 4, 6, 12, and 24 hours, 10 μl cell counting kit-8 reagent (CCK-8; 7Sea Biotech, China) was added to the culture medium of each well. After incubation at 37°C for 2 h, the absorbance was detected at 450 nm by a microplate reader (Thermo Fisher Scientific, USA) and cell's survival rates were represented through the mean absorbance value of each well minus the blank value.

2.6. Transwell Chamber Migration Assay. Six-well transwell chamber with 8 mm filter inserts (Corning, China) was used to observe bEnd.3 migration. Cells were digested into suspensions with trypsin; the upper chambers were filled with 2×10^5 cells/ml containing DMEM (Gibco) and 1% FBS (Gibco), and the lower chambers were filled with 600 μl medium containing 10% FBS (Gibco) and MG-Exos (100 μg/ml) or Vehicle. After culturing the cells in completed medium with 500 μM H_2O_2 for 24 h, the nonmigrated cells on the upper chambers were removed, and the migrated cells on the lower surface were fixed in 4% PFA for 20 min. And then, the migrated cells were stained with 0.1% crystal violet for 20 min and quantified by counting six random fields.

2.7. Scratch Wound Migration Assay. bEnd.3 cells were seeded on six-well plates until confluence reaching at 90%. The monolayer cells were scratched in a cross shape using a P-200 tip. After cultured in completed medium with 500 μM H_2O_2 for 0 h, 6 h, and 12 h after scratching, the wound width was recorded with Microscope (Primovert, Zeiss, Germany). The ImageJ (National Institutes of Health) was used for measuring the distance between the sides of the scratch in six random fields.

2.8. Tube Formation Assay. 50 μl precooled growth factor-reduced Matrigel (BD) was added to a 96-well plate. Then, bEnd.3 (2×10^4 cells/well) were seeded onto the Matrigel and treated with MG-Exos or Vehicle. After cultured in the medium with 500 μM H_2O_2 for 12 h, the network formation was assessed using a bright-field microscope (Zeiss). The ability to form capillary-like structures was quantified by determining the number of branch points, tubule lengths, and loops in six randomly chosen microscopic fields using the ImageJ software.

2.9. Measurement of Intracellular ROS Levels. The level of intracellular reactive oxygen species (ROS) was measured using Reactive Oxygen Species Assay Kit (DCFH-DA) (Solarbio, China), in which the intensity of fluorescence is proportional to ROS level. bEnd.3 were first seeded on 24-well plates. When they reached 70 to 80% confluence, they were cultured with the completed medium with 500 μM H_2O_2 and MG-Exos or Vehicle for 24 h. Then, DCFH-DA was added and incubated for 30 min in dark at 37°C, followed by a fixation with 4% PFA for 20 min. After washing with PBS, cells were stained with DAPI. Fluorescence was measured using a fluorescence microscope (Zeiss Apotome 2).

In vivo, the spinal cords of contusion or sham were collected and digested into the supernatant by using the Collagenase/Dispase (Sigma). After red blood cell lysis and cells counts, DCFH-DA was added and incubated for 30 min in dark at 37°C. After washing three times with the medium, the cells were resuspended in PBS. Fluorescence was detected at 525 nm by a microplate reader (Thermo Fisher Scientific).

2.10. Animals and Surgical Procedures. All animal protocols were approved by the Ethics Committee of Central South University (CSU) for Scientific Research. Experiments were conducted using 8-12-week-old female C57BL/6 (Charles River) mice. An SCI model of female mice was established, as previously described [32]. In brief, female mice were deeply anesthetized with ketamine/xylazine/acepromazine (50 : 5 : 1 mg/kg) by intraperitoneal (i.p.) injection. After a laminectomy used to expose the spinal cord at the 10th thoracic vertebrae (T10), moderate contusion injuries of the spinal cord were created using a modified Allen's weight drop apparatus (10 g weight at a vertical height of 20 mm, 10 g × 20 mm). Mice in the sham group were subjected to laminectomy without contusion. Bladders of animal were manually voided twice per day until full voluntary or autonomic voiding was obtained, and antibiotic (penicillin sodium; North China Pharmaceutical, China) was administered daily for 3 days postsurgery.

Mice were randomly and blindly divided into two groups ($n = 8$/group for each time point) and treated with 200 μl of Vehicle or 200 μg of MG-Exos in 200 μl, administered postsurgery by tail vein injection.

The functional recovery from SCI was measured at different time points through locomotor behavioral assessments, electrophysiological test, and H&E, as well as immunofluorescence analyses.

2.11. Evaluation of the Locomotive Function. To evaluate the locomotive function of hindlimbs, BMS (Basso Mouse Scale) system was used before surgery and 1, 3, 7, 14, 21 and 28 days post-SCI [33]. The BMS ranges from 0 (complete paralysis) to 9 (normal locomotion) points. The 11-point BMS subscore which included frequency of plantar stepping, coordination, paw position, trunk stability, and tail position was also assessed and supplemented the main scale. Each mouse was observed 5 min, and the average BMS scores and subscores were recorded by two well-trained investigators who were familiar with the BMS scores and blinded to the experimental design.

2.12. Neuroelectrophysiology. To evaluate movement recovery after SCI, the MEPs (motor evoked potentials) of hindlimbs were recorded by electromyography conducted at 4 weeks post-SCI as described in our previous research [34]. Briefly, after anesthesia, the stimulating electrodes were secured onto the surface of the skull corresponding to the motor area of the cerebral cortex, while the recording electrodes were inserted into the tibialis anterior muscle of the contralateral hindlimb. The reference electrode was placed into subcutaneous tissue between the stimulating and recording electrodes. The mean MEP values (including amplitude and latency period) were captured before surgery and 4 weeks postsurgery.

2.13. Quantitative Real-Time PCR (qRT-PCR) Analysis. Total RNA was extracted using TRIzol Reagent (Invitrogen, USA). Single-strand cDNA was reverse transcribed from 1 μg total RNA from each sample using GoScript™ Reverse Transcription System (Promega Corporation, USA) according to the manufacturer's instructions. qRT-PCR was performed by GoScript™ qPCR Master Mix (Promega) using a BRYT Green master mix. All reactions were processed and analyzed on a real-time PCR System (FTC-3000, Funglyn Biotech Inc., Canada). The expression level of target genes was normalized to GAPDH, and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences used for qRT-PCR were shown in Table 1.

2.14. Western Blotting. Cells and exosomes were lysed by RIPA buffer containing protease and phosphatase inhibitor (Solarbio). After centrifugation at 10,000×g for 10 min, the supernatant was collected, and the protein concentration of which was detected using the BCA Protein Assay Kit (Solarbio). Then, the supernatant was diluted with protein loading buffer (5×; Beyotime Biotechnology, China) and heated at 100°C for 10 min. Proteins were separated by SDS-PAGE gels 10~12%, transferred to polyvinylidene fluoride membrane (Millipore). The membranes were blocked with 5% milk in TBST (Tris-buffered saline, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 60 min and then incubated with primary antibodies at 4°C overnight. Primary antibodies and dilutions were used as follows: rabbit anti-NOX2 (1 : 1,000), rabbit anti-Nrf2 (1 : 1,000), rabbit anti-keap1 (1 : 2,000), rabbit anti-HO-1 (1 : 1,000), rabbit anti-TSG101 (1 : 2,000), rabbit anti-CD63 (1 : 1,000), and rabbit anti-CD9 (1 : 1,000). Blots were washed with TBST and incubated with peroxidase-conjugated goat anti-rabbit IgG (1 : 5,000). Equal loading of proteins was assessed using a rabbit anti-β-actin (1 : 5,000). All primary antibodies purchased from Proteintech (China). The immunoreactive bands were visualized by using the Enhanced Chemiluminescence Reagent (Millipore) and imaged by ChemiDoc XRS Plus luminescent image analyzer (Bio-Rad, England).

2.15. Flow Cytometry. Microglia were first blocked Fc receptors with anti-mouse CD16/CD32 antibody (BD, USA) for 15 min at 4°C in FACS buffer (PBS with 2% FBS and 2 mM EDTA) and then surface stained with antibodies for CD45 (clone 30-F11, Alexa Fluor 700), F4/80 (clone 90, FITC), CD11b (clone M1/70, APC), and CD68 (clone FA-11, FITC) or isotype control for 30 min at 4°C. After washed three times with FACS buffer, cells were stained with DAPI (BD) in FACS buffer to discriminate dead cells and run on a BD FACS Canto II (BD). Data were analyzed with Flow Jo (BD). All antibodies were purchased from Biolegend (USA).

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

NQo1	Forward primer	ATGGGAGGTGGTCGAATCTGA
	Reverse primer	GCCTTCCTTATACGCCAGAGATG
Gclc	Forward primer	GGGGTGACGAGGTGGAGTA
	Reverse primer	GTTGGGGTTTGTCCTCTCCC
Cat	Forward primer	AGCGACCAGATGAAGCAGTG
	Reverse primer	TCCGCTCTCTGTCAAAGTGTG
Gsx1	Forward primer	CTTCCCTCCCTTCGGATCG
	Reverse primer	GTCCACAGAGATGCAGTGAAA

2.16. Immunofluorescence. To collect the spinal cords after contusion injury or sham operation mice, mice were anesthetized and then rapidly perfused transcardially with 0.9% saline, followed by 4% PFA. Then, the spinal cords were cryoprotected in 30% sucrose for 3 d at 4°C, before being sectioned. Serial cryostat sections (14 mm thick) were obtained. For immunofluorescent staining, the sectioned slices were permeabilized with 0.3% Triton X-100 in PBS for 30 min and then blocked with 5% BSA for 1 h. After incubation with goat anti-CD31 Alexa Fluor 488-conjugated antibody (1 : 100, R&D, USA), rabbit anti-ki67 (1 : 200, Invitrogen, USA), and rabbit anti-Nrf2 (1 : 200, Proteintech) overnight at 4°C, the sectioned slices were incubated with secondary antibodies anti-rabbit Alexa Fluor 488 or Alexa Fluor 568 (1 : 400, Abcam) for 1.5 h. The slices were mounted with Fluoroshield™ with DAPI (GeneTex Inc.). Signals were analyzed by a fluorescence microscope (Zeiss Apotome 2). Positive cells were quantified using the ImageJ software.

Cell samples were fixed with 4% PFA for 20 min, permeabilized with 0.2% Triton X-100 for 15 min, and blocked with 5% BSA for 30 min and then incubated with primary antibodies overnight at 4°C, followed by secondary antibody incubation for 1.5 h. After washing with PBS, cells were stained with DAPI.

2.17. Statistical Analysis. The results were statistically analyzed with SPSS 22.0 (SPSS, Inc.). All data were presented as the means ± standard deviation (SD). Statistical analysis of multiple-group comparison was performed by one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. Values of p less than 0.05 were considered statistically significant.

3. Result

3.1. Identification of Microglia and MG-Exos. Microglia colonies appeared approximately 10-12 days after initial plating. The cells exhibited a typically elongated, either bipolar

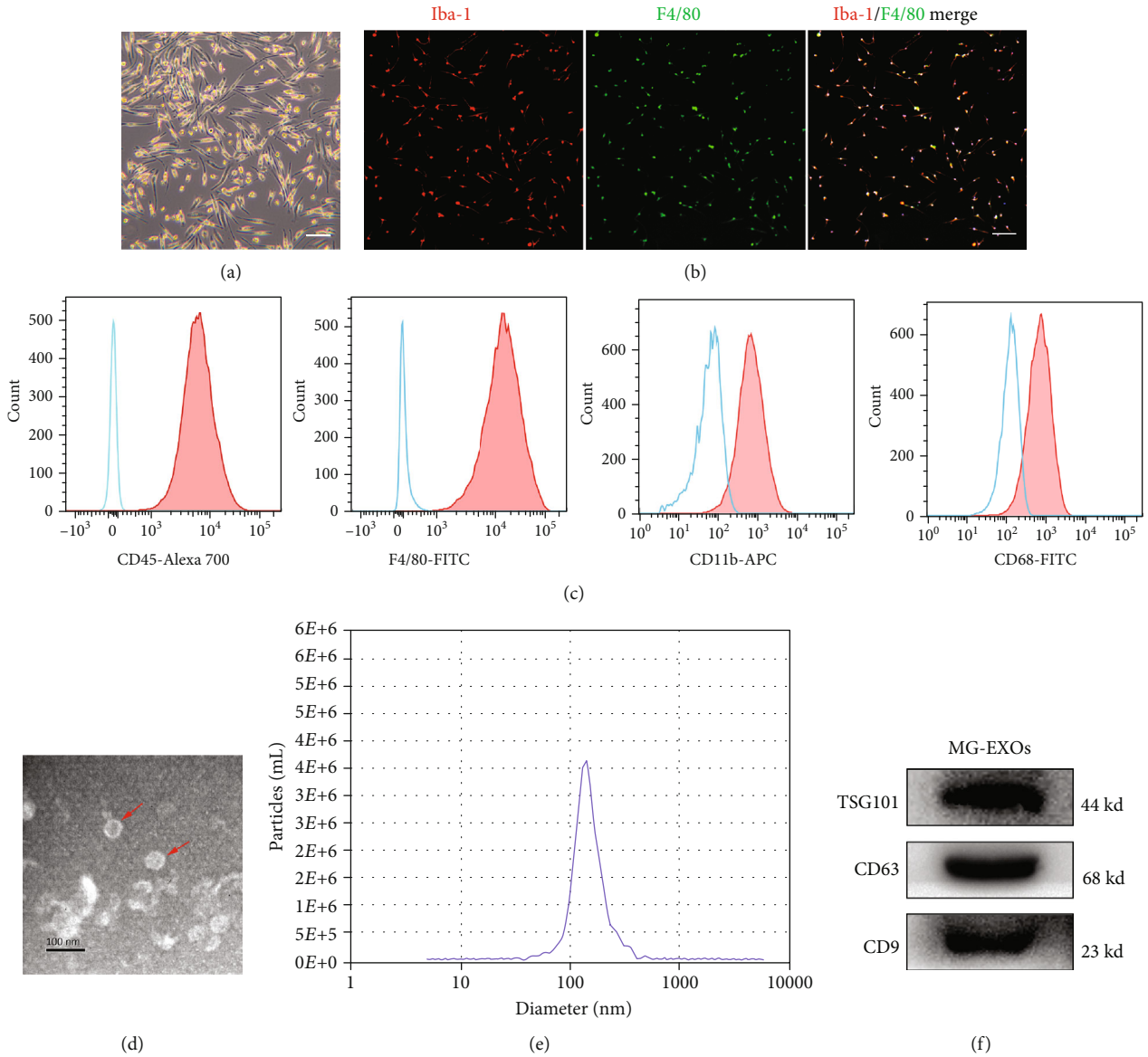


Figure 1: Identification of microglia and MG-Exos. (a) The morphology of microglia. Scale bars = 100 μm. (b) Representative immunofluorescence images of microglia. Iba-1 (red) and F4/80 (green), scale bars = 100 μm. (c) Flow cytometry analysis of the cell markers of microglia. The isotype controls are illustrated as blue dashed curves, and the test samples are illustrated as solid red curves. (d) Representative images of MG-Exo morphology detected by transmission electron microscopy (TEM). Scale bar = 100 nm. Red arrows indicate exosomes. (e) Size distribution assessed by nanoparticle tracking analysis (NTA). (f) Western blotting analysis of specific exosomal surface markers.

or unipolar, morphology (Figure 1(a)). We identified the cultured microglia by immunofluorescence costaining of Iba-1 and F4/80 (Figure 1(b)). Flow cytometry analysis revealed that microglia surface markers were highly positive including CD45, F4/80, CD11b, and CD68 (Figure 1(c)). All these features were consistent with the previous studies [35]. TEM and NTA were used to identify particles purified from microglial cells. As shown in Figures 1(d) and 1(e), the nanoparticles exhibited a cup- or sphere-shaped morphology with a size of approximately 80~150 nm. Characteristic biomarkers of exosomes, including TSG101, CD63, and CD9, were also verified by western blotting (Figure 1(f)).

3.2. MG-Exos Were Taken Up by Endothelia Cells, Inhibited ROS In Vivo and Vitro. Oxidative stress is regarded as a critical factor that contributes to the development of a poor microenvironment in SCI repair [4, 36]. To examine the role of MG-Exos played against the toxic effects of oxidative stress, we determined whether MG-Exos could be transferred into endothelia cells firstly. As shown in Figure 2(a), PKH67-labeled MG-Exos (green) were transferred into the perinuclear region of bEnd.3 after 12 h incubation. For functional assays, MG-Exos and an equal volume of Vehicle were injected through the tail vein for 3 consecutive days post-SCI, respectively. And the ROS level of the epicenter (injury

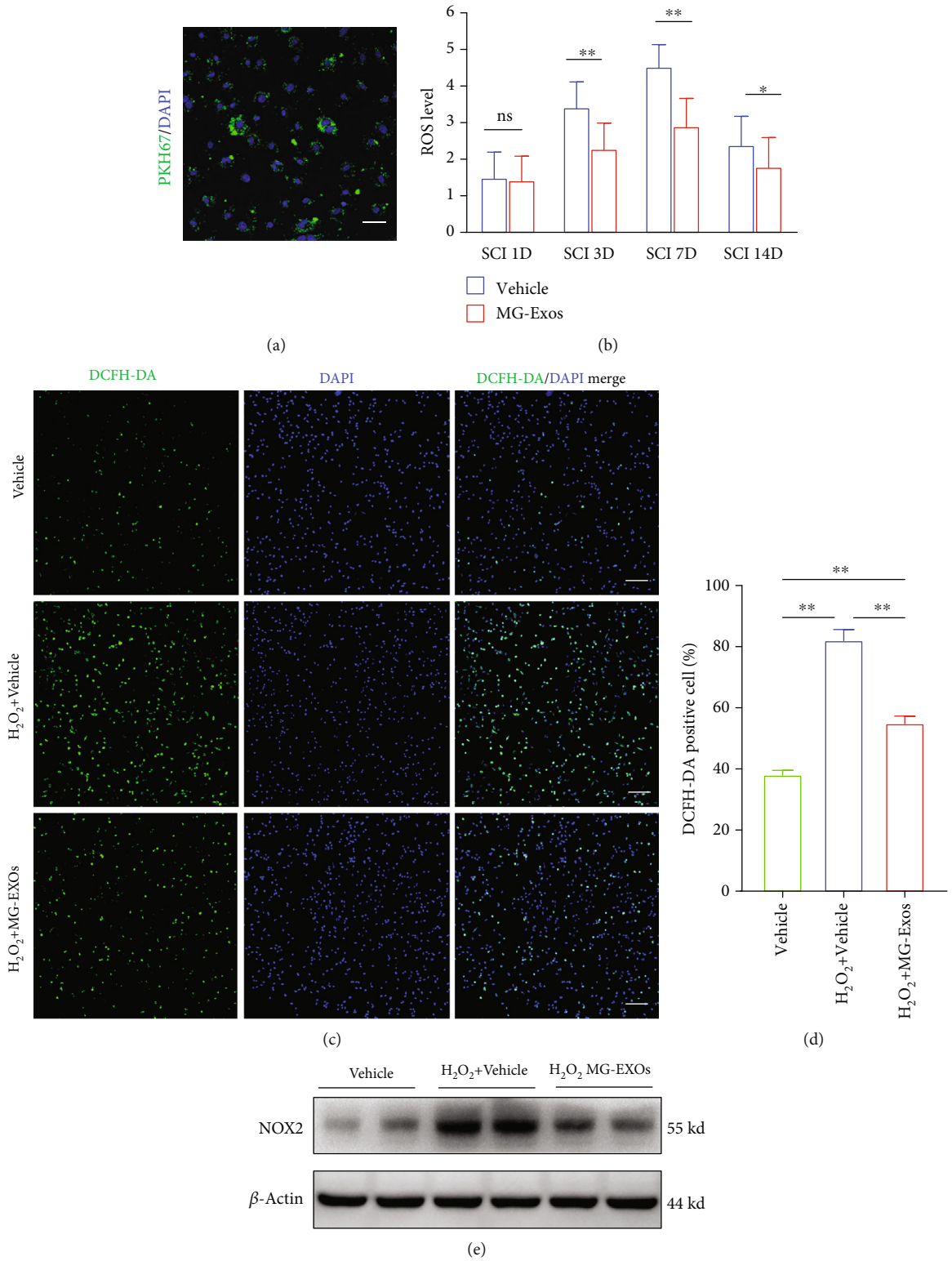
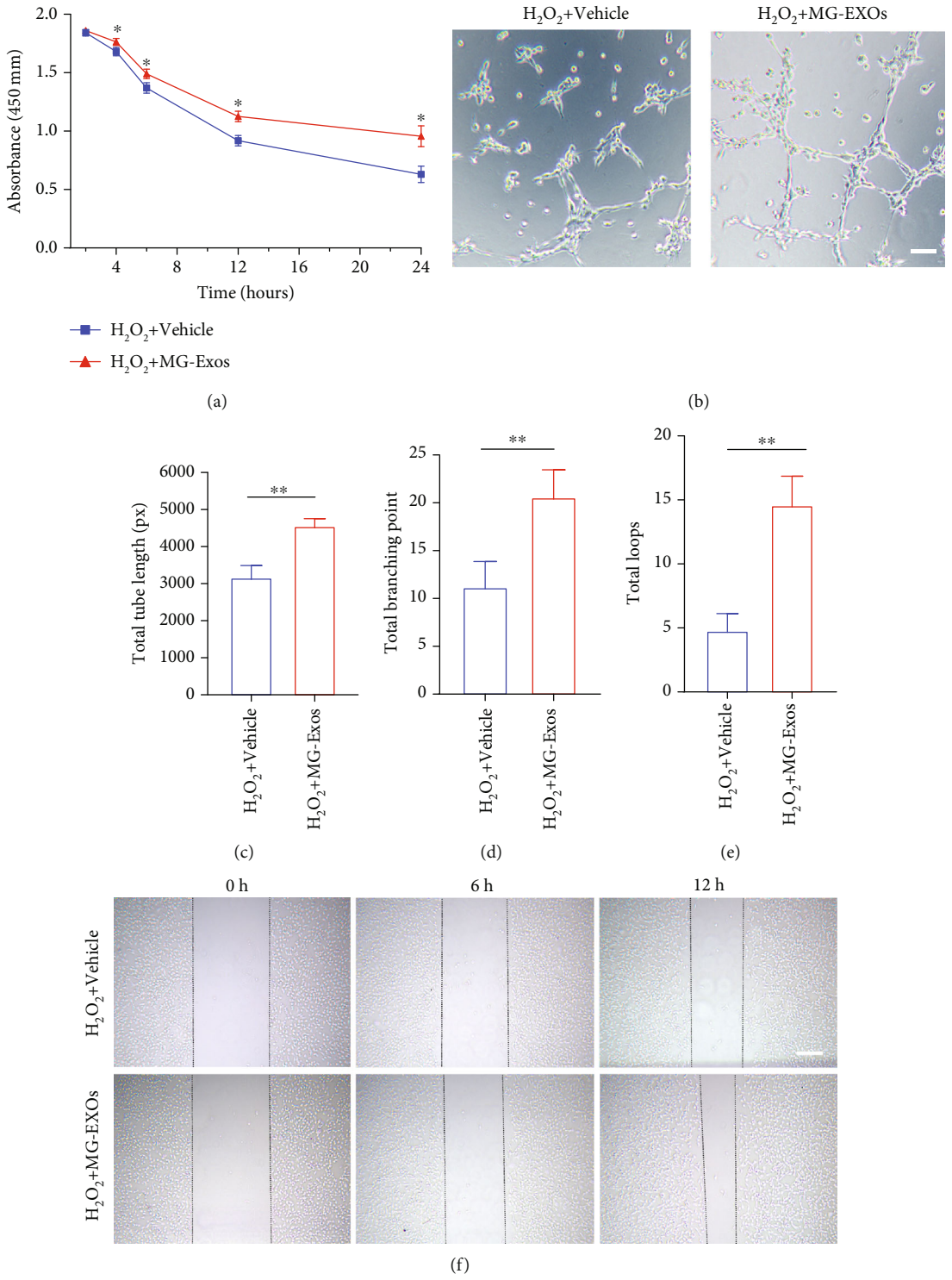


Figure 2: MG-Exos were internalized into endothelia cells and inhibited ROS *in vivo* and *in vitro*. (a) Fluorescence microscopy analysis of PKH67-labeled MG-Exos taken up by bEnd.3. Scale bar = 50 μm. (b) The ROS level of the epicenter of the spinal cord's injury area per group was detected by the DCFH-DA assay throughout the 14-day period post-SCI. (c) Representative fluorescence images of the ROS levels of bEnd.3 treated with H_2O_2 plus MG-Exos or Vehicle. DCFH-DA (green) and nuclei (blue); scale bar = 100 μm. (d) Quantitative analysis of the number of DCFH-DA positive cells in (c). (e) Western blotting analysis of the protein levels of NOX2 in bEnd.3 treated with MG-Exos or Vehicle when exposed to H_2O_2-induced oxidative stress. The data are presented as the means ± SD; $n = 6$ per group. $^*p < 0.05$ and $^{**}p < 0.01$ compared with different treatment groups.



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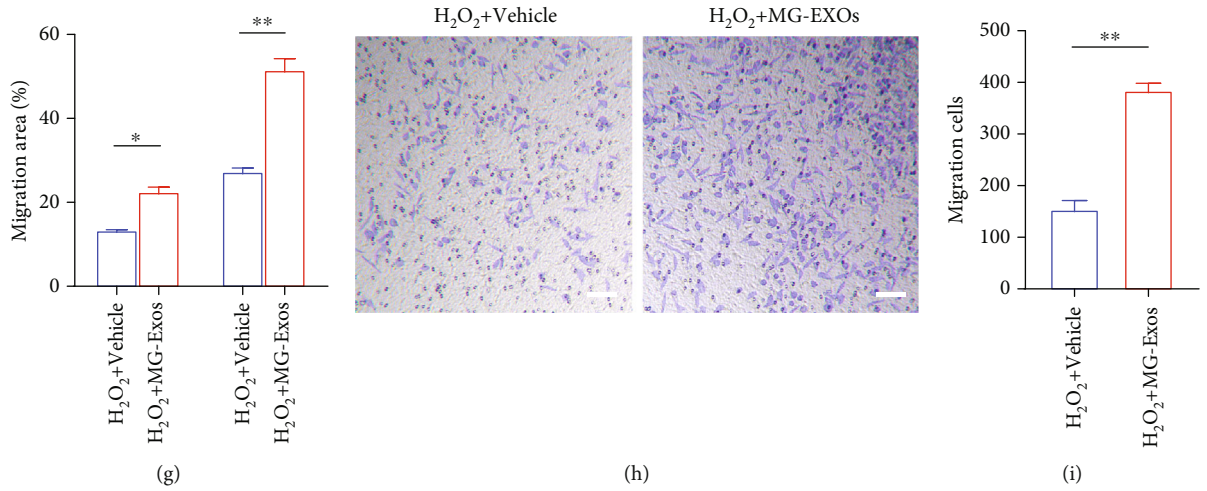


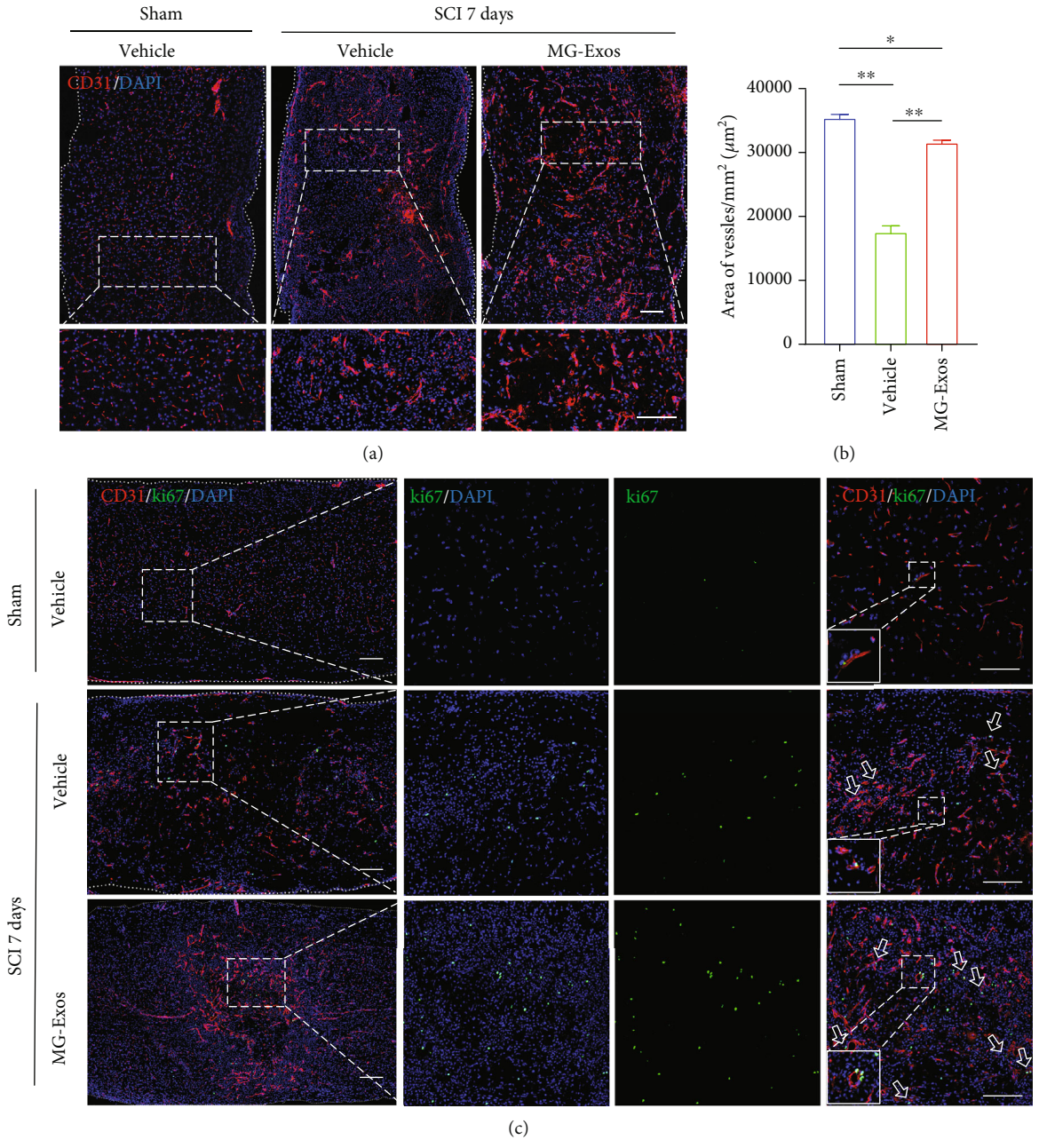
FIGURE 3: MG-Exos promote survival and function of endothelial cells *in vitro*. (a) CCK-8 analysis of the survival rate of bEnd.3 treated with MG-Exos or Vehicle when exposed to H_2O_2-induced oxidative stress. (b) Representative images of bEnd.3 tube formation *in vitro* after H_2O_2 plus Vehicle or MG-Exo treatment. Scale bar = 50 μm. (c–e) Quantitative evaluation of the total tube length, total branching points, and total loops in (b). (f) Representative images of bEnd.3 migration in the H_2O_2 plus Vehicle or MG-Exo treatment groups in the scratch assay. Scale bar = 250 μm. (g) Quantification of the percentage of migration area in (f). (h) Representative images of transwell experiment in the H_2O_2 plus Vehicle or MG-Exo treatment groups. Scale bar = 100 μm. (i) Quantitative evaluation of the number of migration cells in (h). The data are presented as the means ± SD; $n = 6$ per group. $^*p < 0.05$ and $^{**}p < 0.01$ compared with different treatment groups.

site) of the spinal cord was detected by the DCFH-DA assay. The results revealed that the ROS level of the MG-Exos-treated mice was remarkedly decreased in comparison with that in Vehicle-treated mice starting at 3 days post-SCI till 14 days after injury, as shows in Figure 2(b). We next assessed whether the protective effects of MG-Exos could reduce the ROS level in endothelia cells. Fluorescence microscope of DCFH-DA was performed *in vitro*. As shown in Figures 2(c) and 2(d), the level of ROS significantly increased when exposed to the H_2O_2 which induced a high level of oxidative stress but decreased after MG-Exo treatment. Further, the protein level of NADPH oxidase 2 (NOX2), a marker of oxidative stress, in both treatment groups (MG-Exos and Vehicle) was analyzed by western blotting (Figure 2(e)). Accordingly, the levels of ROS and the expression of NOX2 detected *in vivo* and i*n vitro* confirm the protective effect of MG-Exos on endothelia cells against oxidative stress.

3.3. MG-Exos Promote Survival and Function of Endothelial Cells In Vitro. To investigate whether the MG-Exos improve endothelial cells' survival and functional recovery when exposed to H_2O_2-induced oxidative stress, there were several functional assays performed. CCK-8 assay showed that the survival rate of bEnd.3 was markedly elevated in response to MG-Exo stimulation (Figure 3(a)). The tube formation assay on Matrigel is an *in vitro* model of angiogenesis. As shown in Figure 3(b), bEnd.3 treated with MG-Exos showed a higher number of capillary-like structures compared to the Vehicle-treated group when both groups were exposed to H_2O_2. Quantitative measurements revealed that the total tube length, total branching points, and total loops in the MG-Exo-treated group were all drastically higher than that in the Vehicle group (Figures 3(c)–3(e)). MG-Exo treatment also caused a remarkable increase in bEnd.3 migration compared to the control groups under oxidative condition, as evidenced by the scratch wound assay (Figures 3(f) and 3(g)) and transwell migration assay (Figures 3(h) and 3(i)). These findings indicate that MG-Exos elevated the survival rate and promote the functional recovery of endothelial cells under H_2O_2-induced oxidative condition.

3.4. MG-Exos Promote Vascular Regeneration Post-SCI In Vivo. We then evaluated the role of MG-Exos played in angiogenesis in a contusive SCI model. Seven days postinjury, a time point at which the proliferative capacity of the blood vessels reached the climax was chosen to evaluate the endothelia cell proliferation [14]. Immunofluorescence analysis revealed that the area of CD31-positive cells in MG-Exo treatment significantly increased in comparison with that in Vehicle-treated mice at 7 days post-SCI (Figures 4(a) and 4(b)). We also performed CD31 and ki67 costaining to test the proliferation of microvascular endothelia cells in the injury site of spinal cord. The result revealed that CD31 and ki67 double-positive cells were rarely observed in the Vehicle-treated mice at day 7 post-SCI, while larger numbers of proliferating microvascular endothelia cells appeared in the injury site when treated with MG-Exos (Figures 4(c) and 4(d)). Taken together, our data suggest that MG-Exos could promote angiogenesis *in vivo*.

3.5. MG-Exos Improve Spinal Functional Recovery Post-SCI. To further determine the therapeutic effects of MG-Exos on neurological functional recovery after SCI, histological analysis and behavior tests were performed. H&E staining was carried out to explore the extent of spinal cord repair. As shown in Figures 5(a) and 5(b), the injury area remarkedly decreased in the MG-Exo-treated mice compared to



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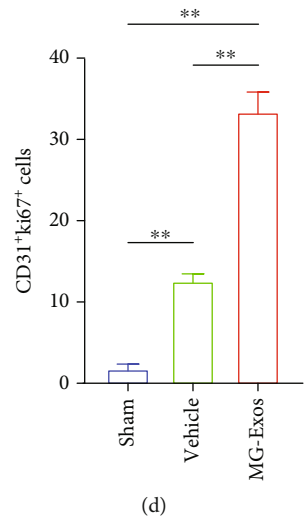

(d)

Figure 4: MG-Exos promote vascular regeneration post-SCI *in vivo*. (a) Representative immunofluorescence images of CD31 blood vessels in the epicenter of spinal cord's injury area in each group at 7 days post-SCI. CD31 (green) and nuclei (blue); scale bar = 200 μm and 50 μm (enlarged view). (b) Quantitative evaluation of the area vessels in (a). (c) Representative immunofluorescence images of CD31 (red) and ki67 (green) blood vessels in the mouse's spinal cord in each group at 7 days post-SCI. Scale bars = 200 μm and 50 μm (enlarged view). White arrows indicate $CD31^+ki67^+$ cells. (d) Quantification of the number of CD31 and ki67 double-positive cells in (c). The data are presented as the means ± SD; $n = 6$ per group. $^*p < 0.05$ and $^{**}p < 0.01$ compared with different treatment groups.

the Vehicle-treated mice at 28 days post-SCI. In consistent with H&E, during the motor function test, the mice treated with MG-Exos showed drastically elevated locomotor recovery after SCI, as indicated by increased BMS scores and BMS subscores starting at 7 days post-SCI till 28 days after injury (Figures 5(c) and 5(d)). In addition, the electrophysiological analysis of motor-evoked potentials (MEPs) indicated the amplitude of the MG-Exo-treated mice significantly increased compared to the Vehicle-treated mice at 28 days post-SCI (Figures 5(e) and 5(f)); moreover, the latent period was shortened in the MG-Exo-treated mice (Figure 5(g)). These results demonstrated MG-Exos had the protective effects on endothelia cells against functional loss in SCI.

3.6. MG-Exos Minimized the Negative Impacts of Oxidative Stress in Endothelia Cells by Modulating the keap1/Nrf2/HO-1 Signaling Pathway. To explore whether the keap1/Nrf2 signaling pathway involved in the endothelia cells' protective process against oxidative stress after MG-Exo treatment, levels of keap1, Nrf2, and HO-1 proteins were measured using immunofluorescence staining and western blotting. As shown in Figures 6(a) and 6(b), immunofluorescence staining indicated that excessive oxidative stress would decrease the expression levels of Nrf2, while MG-Exo treatment reversed the change. In accordance with the immunofluorescence results, the western blotting showed that the protein levels of keap1 in the MG-Exo-treated group after exposed to H_2O_2 were significantly downregulated compared to the Vehicle group; however, the protein levels of Nrf2 and HO-1 were upregulated in the MG-Exo-treated group (Figure 6(c)). qRT-PCR was further carried out in H_2O_2-treated bEnd.3 to validate the mRNA of downstream antioxidative-related genes, which were reported to be closely (*NQo1*, *Gclc*, *Cat*, and *Gsx1*) associated with the keap1/Nrf2 pathway [37–39]. As quantified analysis indicated, the mRNA levels of *NQo1*, *Gclc*, *Cat*, and *Gsx1* were significantly higher in the MG-Exo-treated group (Figures 6(d)–6(g)). These results suggest that MG-Exos inhibit oxidative stress in endothelia cells via modulating the keap1/Nrf2/HO-1 signaling pathway.

4. Discussion

SCI is a worldwide devastating disease of the central nervous system companied by a long-term disability and high mortality [1, 40]. Until now, numerous treatment strategies have been used to ameliorate the neurological regeneration of the spinal cord after SCI, but unfortunately, none have been convincingly effective in improving the neurological function of SCI patients. In our present study, we found that microglia-derived exosomes protected blood vessel against the toxic effects of high oxidative stress and improved spinal cord functional recovery. Our results revealed that MG-Exos could reduce the level of H_2O_2-induced ROS of endothelia cells *in vivo* and the ROS level in the epicentral spinal cord in the mice's SCI model. We further confirmed that MG-Exos could promote endothelia cells' survival and function *in vitro* and new vessel formation *in vivo*. The activation of the keap1/Nrf2/HO-1 signaling pathway was the potential mechanisms underlying the MG-Exo treatment for SCI.

Emerging evidences suggested that microglia could influence the formation of new blood vessels and vascular development in the central nervous system and retina [25, 26]. In spinal cord tissue, microglia and microvascular endothelial cells have a compact anatomical structure. During SCI, microglia would form clusters around the vasculature, and then, phagocytose damaged vascular tissue [23]. Subsequently, they would secrete multiple factors such as VEGF,

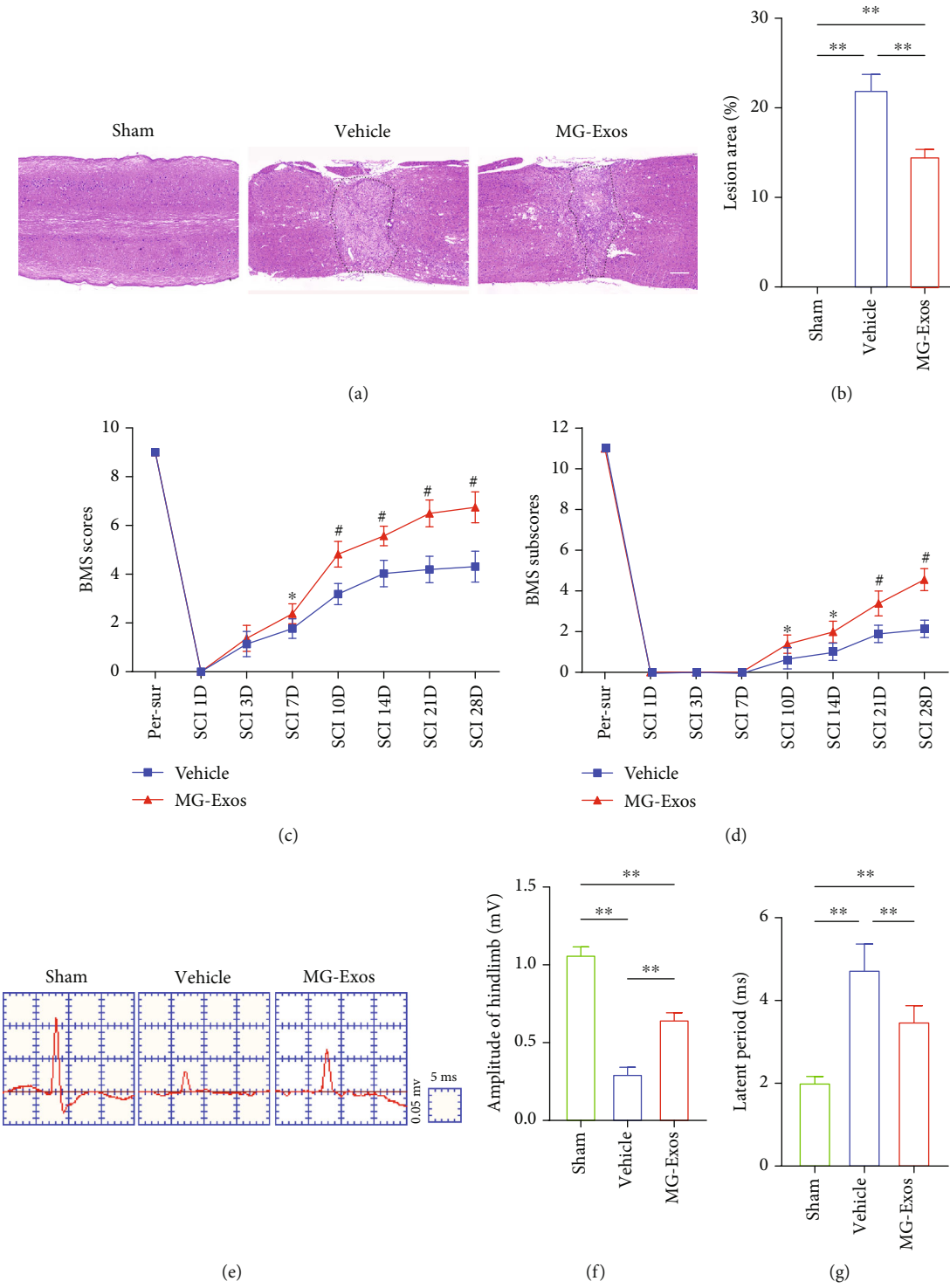

Figure 5: MG-Exos improve spinal functional recovery after SCI. (a) Representative H&E staining of the longitudinal epicenter injury of spinal cord in each group at 28 days post-SCI. Scale bar = 1 mm. (b) Quantification of the lesion area in (a). (c) Distribution of the BMS scores per group after SCI throughout the 28-day period. (d) Distribution of the BMS subscores per group at 0, 1, 3, 7, 14, 21, and 28 days post-SCI. (e) Representative electrophysiological traces in each group at 28 days post-SCI. (f, g) Quantification of the amplitude and latent period of MEPs in (e). The data are presented as the means ± SD; $n = 6$ per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with different treatment groups.

basigin-2, and FGF2, which are known to promote angiogenesis and tissue repair [23, 27]. Meanwhile, microglia-lacking mice, with which some other experiments conducted, were found to have reduced developing retinal vasculature [28]. Theses evidences suggested that microglia could promote the reconstruction of blood vessel following

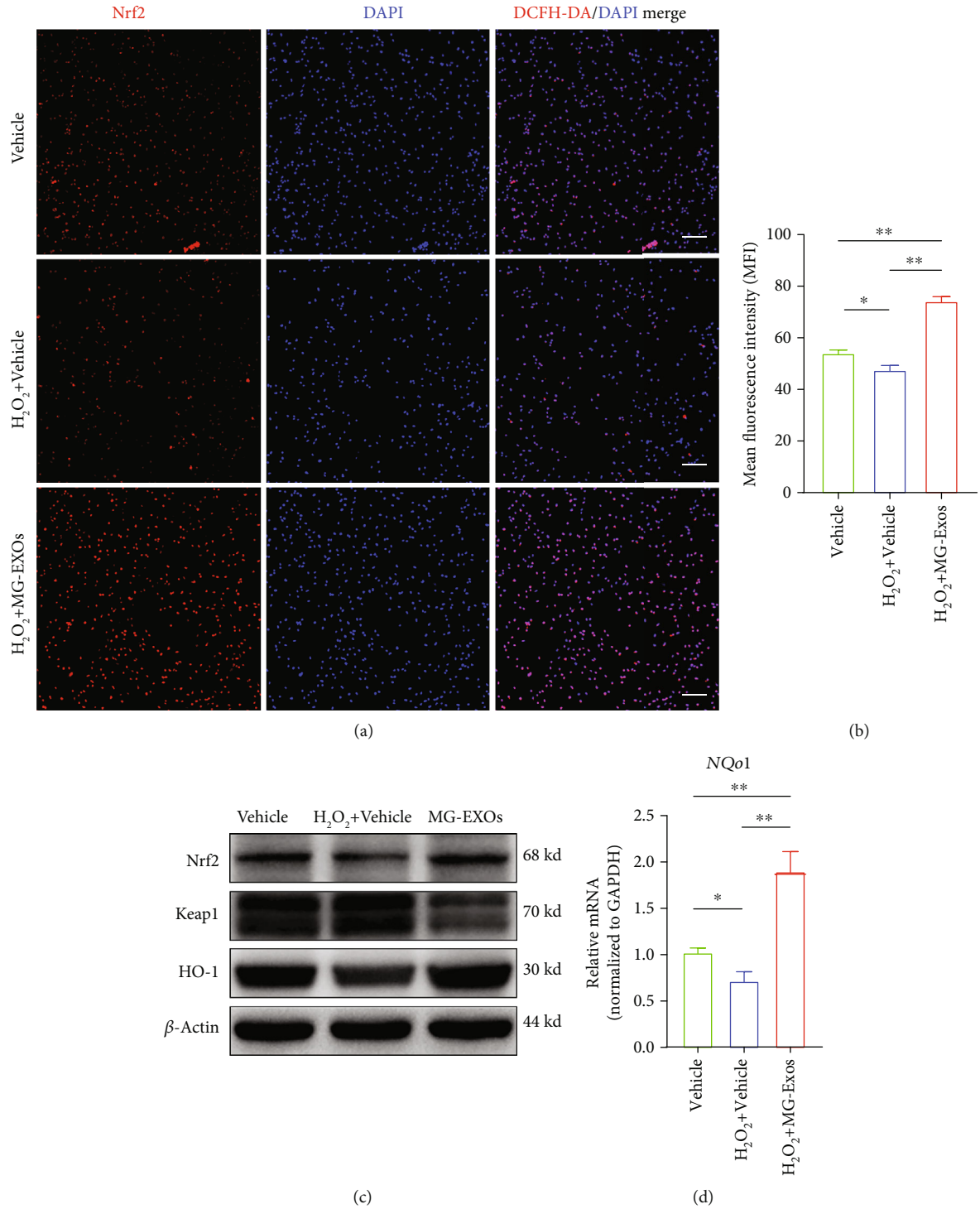

Figure 6: Continued.

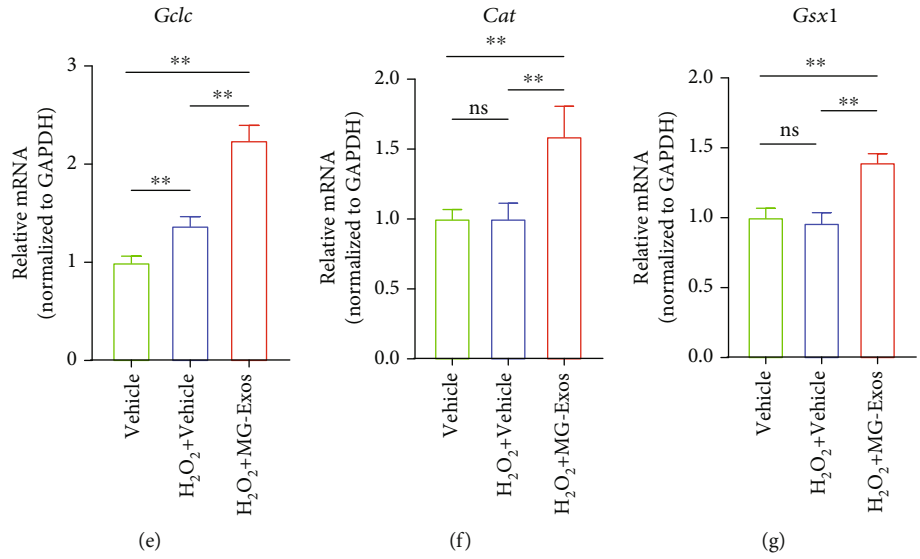

Figure 6: MG-Exos protect endothelia cells against the oxidative effects by modulating the keap1/Nrf2/HO-1 signaling pathway. (a) Representative immunofluorescence images of the Nrf2's expression of bEnd.3 after Vehicle or MG-Exo treatment when exposed to H_2O_2-induced oxidative stress. Scale bar = 100 μm. (b) Quantification of mean fluorescence intensity (MFI) in (a). (c) Western blotting analysis of the protein levels of keap1, Nrf2, and HO-1 in H_2O_2 plus Vehicle or MG-Exo treatment groups. (d–g) qPCR verification of the mRNA levels of downstream antioxidative-related genes including *NQo1*, *Gclc*, *Cat*, and *Gsx1* per group when exposed to H_2O_2-induced oxidative stress. The data are presented as the means ± SD; $n = 6$ per group. $^*p < 0.05$ and $^{**}p < 0.01$ compared with different treatment groups.

central nervous system injury. Moreover, numerous studies have shown that mesenchymal stem cells (MSCs) were mainly relied on their paracrine function rather than their capacity for differentiation to repair tissue damage [41, 42]. This means that exosomes could perform most of the functions of their parental cells. Therefore, we choose microglia exosomes to perform our study.

As we know, neurovascular communication is essential for the homeostasis and well-functioning of the neuronal compartments in the central nervous system [43]. The researches of neurovascular opened a new perspective for current studies of repairing the damaged central nervous system. The reconstruction of damaged neural networks after SCI needed microvasculature to provide nutrients and maintain their homeostasis [14]. Furthermore, the newborn microvasculature could provide the scaffolds for regenerating axons which might grow along the new vessels; otherwise, the abnormalities in angiogenesis would delay neural tissue regeneration [12]. Yuan et al. have reported that pericyte-derived exosomes could improve the endothelial ability of regulating blood flow and then could promote the recovering degree of neurological function after SCI [44]. As shown in our previous studies, the exosomes derived from human urine stem cells could enhance spinal cord functional recovery via promoting vascular regeneration post-SCI [45]. Thus, promoting angiogenesis and restoring blood flow to the injured cord may provide an essential foundation for spinal cord repair and recovery. In our current study, we observed that MG-Exos could drastically increase the number of newly formed blood vessels and could significantly improve the endothelial cells' proliferative ability in the epicentral injury of spinal cord. Our neurological function assays also revealed that MG-Exos could promote the recovery of neurological function post-SCI. These results suggest that the profunction recovering action of MG-Exos is likely attributable to their stimulatory effects on endothelial angiogenesis.

On the other hand, the oxidative stress along with the generating of free radicals drastically leveled up following SCI. They were the main pathological factors of secondary damage, and they also could lead to endothelial dysfunction and the limited endogenous repair of microvasculature during SCI [10]. ROS, a main form of oxidants, could cause lipid peroxidation, protein inactivation, DNA fragmentation, and ultimately cells' dysfunction or even death. Consistent with results of previous researches [22], the increase in levels of oxidative stress products (ROS) and NOX2, a marker of oxidative stress, was found in both the H_2O_2-induced bEnd.3 cells and the SCI model. Treatment with MG-Exos was found to significantly decrease the ROS production and the expression level of NOX2 in the H_2O_2-induced bEnd.3 cells as well as the SCI model. *In vitro*, functional assays of endothelia cells revealed that MG-Exos markedly improved the survival and function of endothelial cells when being exposed to H_2O_2-induced oxidative stress. These findings indicate that MG-Exos have the ability to resist oxidative stress.

To investigate the detailed molecular mechanism of antioxidation, the keap1/Nrf2/HO-1 signaling pathway was evaluated. The activity of Nrf2 was crucial to regulate intracellular oxidative stress status [17, 46]. In normal physiological conditions, Nrf2 anchored in the cytoplasm by keap1 which could promote the ubiquitination of Nrf2. Then, the ubiquitinated Nrf2 would be rapidly degraded by proteasome [47]. When cells were exposed to ROS or free radicals, Nrf2 dissociated from keap1 and quickly transferred into the

nucleus and then binded to the antioxidant response element (ARE), exerting antioxidation through promoting the transcription of its downstream antioxidative-related genes, such as Heme Oxygenase-1 (HO-1), NAD (P) H quinone oxidoreductase 1 (NQo1), Glutamate-cysteine Ligase Catalytic subunit (Gclc), Catalase (Cat), and GS Homeobox 1 (Gsx1) [17]. In our present study, we observed that the protein levels of Nrf2 and HO-1 were markedly decrease in H_2O_2-induced bEnd.3 cells, but keap1's expression had no change. These findings were consistent with previous research results. However, treatment with MG-Exos significantly increased the levels of Nrf2 and HO-1 proteins. Also, the expression of keap1 was remarkedly downregulated in H_2O_2-induced bEnd.3 cells. We further validated that the expression of downstream antioxidative-related factors of keap1/Nrf2/HO-1, including *NQo1*, *Gclc*, *Cat*, and *Gsx1*, had varying degrees of elevation *in vitro*. These results indicated that MG-Exos could exert an antioxidant function through the keap1/Nrf2/HO-1 signaling pathway.

5. Conclusions

In conclusion, our results confirmed that MG-Exos could exert their influences as antioxidants against oxidative stress via activating the keap1/Nrf2/HO-1 pathway. Moreover, MG-Exos promoted the survival as well as functions of endothelia cells and the recovery degrees of neurological function *in vitro* and *vivo*. Therefore, MG-Exos may be able to protect endothelia cells from oxidative stress and promote their restoration, thus enhance the spinal cord functional recovery during SCI treatment.

Data Availability

The data used to support the findings of this study are included in the article. The authors stated that the data underlying the findings of this manuscript is available to share.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

JZH and HBL designed and supervised the study; WP, LYW, YDL, and YX did the experiments; WP, LYW, and TMH analyzed the data; WP and LYW wrote the paper; WP, LYW, and JZH scientifically revised the manuscript. All authors contributed to and approved the manuscript. Wei Peng and Liyang Wan contributed equally to this work.

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Retraction

Retracted: Platelet-Rich Plasma-Derived Exosomal USP15 Promotes Cutaneous Wound Healing via Deubiquitinating EIF4A1

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope
(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
(4) Inappropriate citations
(5) Incoherent, meaningless and/or irrelevant content included in the article
(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] Y. Xu, Z. Lin, L. He et al., "Platelet-Rich Plasma-Derived Exosomal USP15 Promotes Cutaneous Wound Healing via Deubiquitinating EIF4A1," *Oxidative Medicine and Cellular Longevity*, vol. 2021, Article ID 9674809, 14 pages, 2021.

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RETRACTED

Research Article

Platelet-Rich Plasma-Derived Exosomal USP15 Promotes Cutaneous Wound Healing via Deubiquitinating EIF4A1

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Epithelial regeneration is an essential wound healing process, and recent work suggests that different types of exosomes (Exos) can improve wound repair outcomes by promoting such epithelial regeneration. Platelet-rich plasma (PRP) is known to facilitate enhanced wound healing, yet the mechanisms underlying its activity are poorly understood. To explore these mechanisms, we first isolated PRP-derived Exos (PRP-Exos). Using immortalized keratinocytes (HaCaT cells) treated with PBS, PRP, or PRP-Exos, we conducted a series of *in vitro* Cell Counting Kit-8 (CCK-8), EdU, scratch wound, and transwell assays. We then established a wound defect model *in vivo* in mice and assessed differences in the mRNA expression within these wounds to better understand the basis for PRP-mediated wound healing. The functions of PRP-Exos and USP15 in the context of wound healing were then confirmed through additional *in vitro* and *in vivo* experiments. We found that PRP-Exos effectively promoted the *in vitro* proliferation, migration, and wound healing activity of HaCaT cells. USP15 was further identified as a key mediator through which these PRP-Exos were able to promote tissue repair both *in vitro* and *in vivo*. At a mechanistic level, USP15 enhanced the functional properties of HaCaT cells by promoting EIF4A1 deubiquitination. Thus, PRP-Exos and USP15 represent promising tools that can promote wound healing via enhancing epithelial regeneration.

1. Introduction

Chronic wounds are defined as wounds that do not heal appropriately and that persist for three months or longer [1, 2]. Such wounds most commonly arise in the context of diabetes, arterial ischemia, poor venous return, infections, pressure ulcers, or malignant tumors, resulting in pain for the affected patient while also compromising the integrity of barriers that are essential for the prevention of bacterial entry into the human body [3]. As such, chronic wounds can reduce patient quality of life while also imposing a significant economic burden on their families and on society as a whole [4]. There is still a lack of any standard treatments for chronic wounds, although keratinocytes are known to be essential mediators of normal wound healing processes [5, 6]. Targeted efforts to accelerate reepithelialization in the context of wound repair thus represent a key area of ongoing scholarly research.

Platelet-rich plasma (PRP) contains a diverse array of physiologically important growth factors at high concentrations, including transforming growth factor-β (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [7, 8]. Some of these growth factors are closely related to the formation of blood vessels, which is an integral process in the context of tissue regeneration. Indeed, VEGF-producing Schwann cells have been reported to accelerate peripheral nerve repair [9]. As such, PRP has shown great promise as

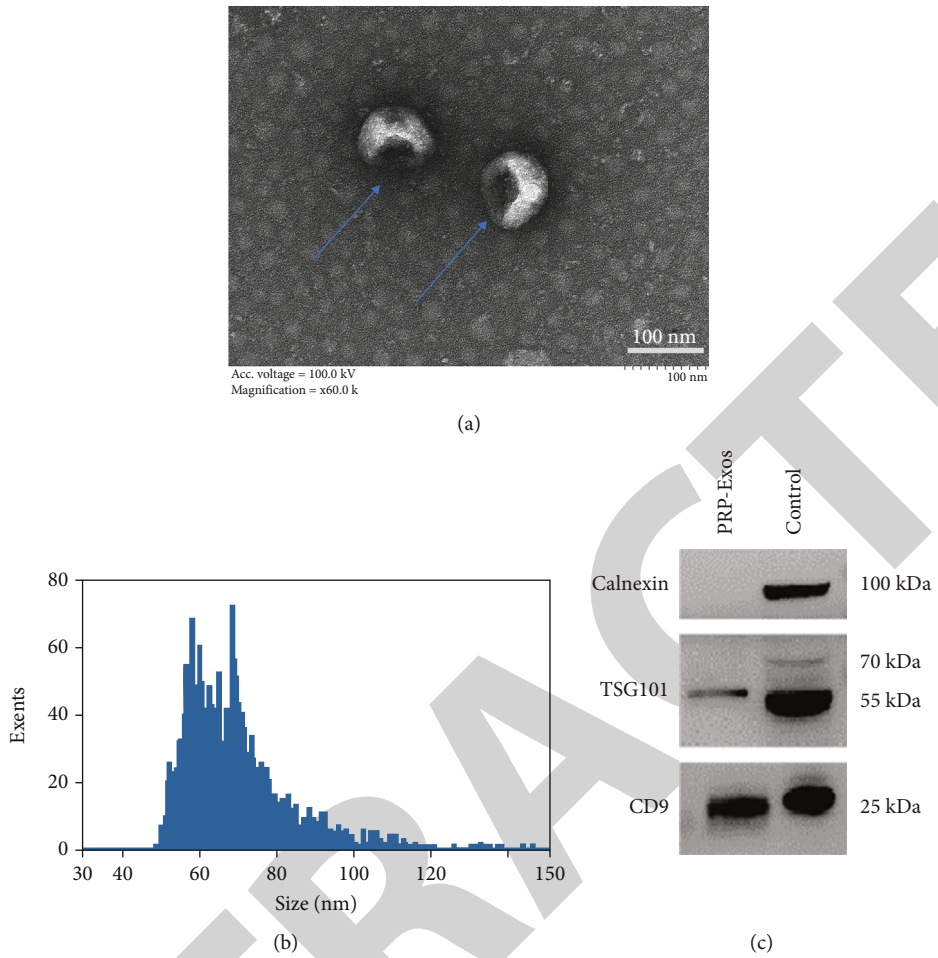

Figure 1: PRP-Exo characterization: (a) TEM images of PRP-Exos; (b) PRP-Exo size distributions, as measured via DLS; (c) Western blotting analyses of proteins within PRP-Exos.

an applied treatment capable of mediating various forms of tissue regeneration. The therapeutic application of PRP has been linked to accelerated angiogenesis and reepithelialization, thereby promoting expedited wound healing [10]. The mechanisms whereby PRP can promote such regeneration, however, are unclear. In addition to the abovementioned growth factors, platelets can produce a variety of different extracellular vesicles, including exosomes [11], which are small vesicles that are approximately 100 nm in diameter (range: 40–160 nm). These vesicles have been a focus of intensive research interest in recent years owing to their essential role as mediators of cell-cell communication, shuttling nucleic acids, proteins, metabolites, and other macromolecules between cells such that they hold promise as tools for treating a range of diseases [12–15]. Owing to the unique biological properties of PRP and of exosomes, further research is warranted to establish the ability of PRP-derived exosomes (PRP-Exos) to shape wound healing processes *in vitro* and *in vivo*.

The present study was therefore designed to evaluate the impact of PRP-Exos on epithelial cell function to explore the mechanistic basis for such activity *in vitro* and *in vivo* in the context of wound healing.

2. Materials and Methods

2.1. Cell Culture. HaCaT cells were purchased from the China Center for Type Culture Collection, Wuhan, China, and were cultured in DMEM containing 10%FBS in a 5% CO_2 incubator at 37°C. Lipofectamine was used to transfect cells with siRNA constructs (50 μmol/L; GenePharma, Shanghai, China).

2.2. PRP Preparation. Mice ($n = 12$) were anesthetized via intraperitoneal injection with 1% sodium pentobarbital, after which blood was collected from the abdominal vena cava into a 1 mL syringe containing 0.1 mL of anticoagulant. The collected blood was transferred into a fresh 1.5 mL tube and stored at 4°C until the collection was completed, at which time samples were spun for 10 min at 100 ×g at 4°C. The supernatant and the layer containing the white blood cells were then collected from each tube and transferred to a new tube, leaving the red blood cell pellet undisturbed. In this case, normal plasma is obtained. These samples were then spun again for 10 min at 600 ×g at 4°C, after which 3/4 of the supernatant was discarded with the remaining sample being gently mixed to yield PRP.

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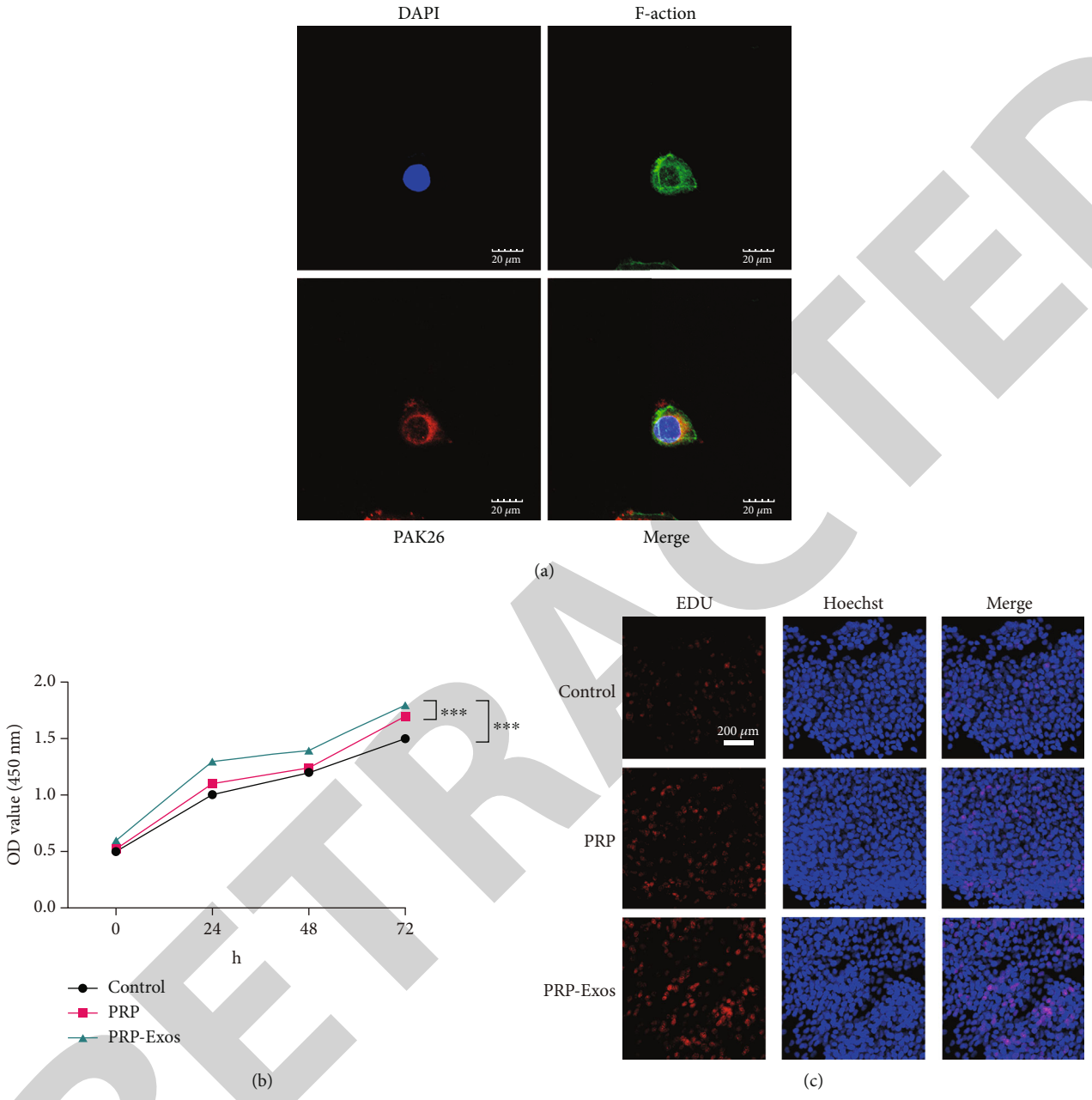

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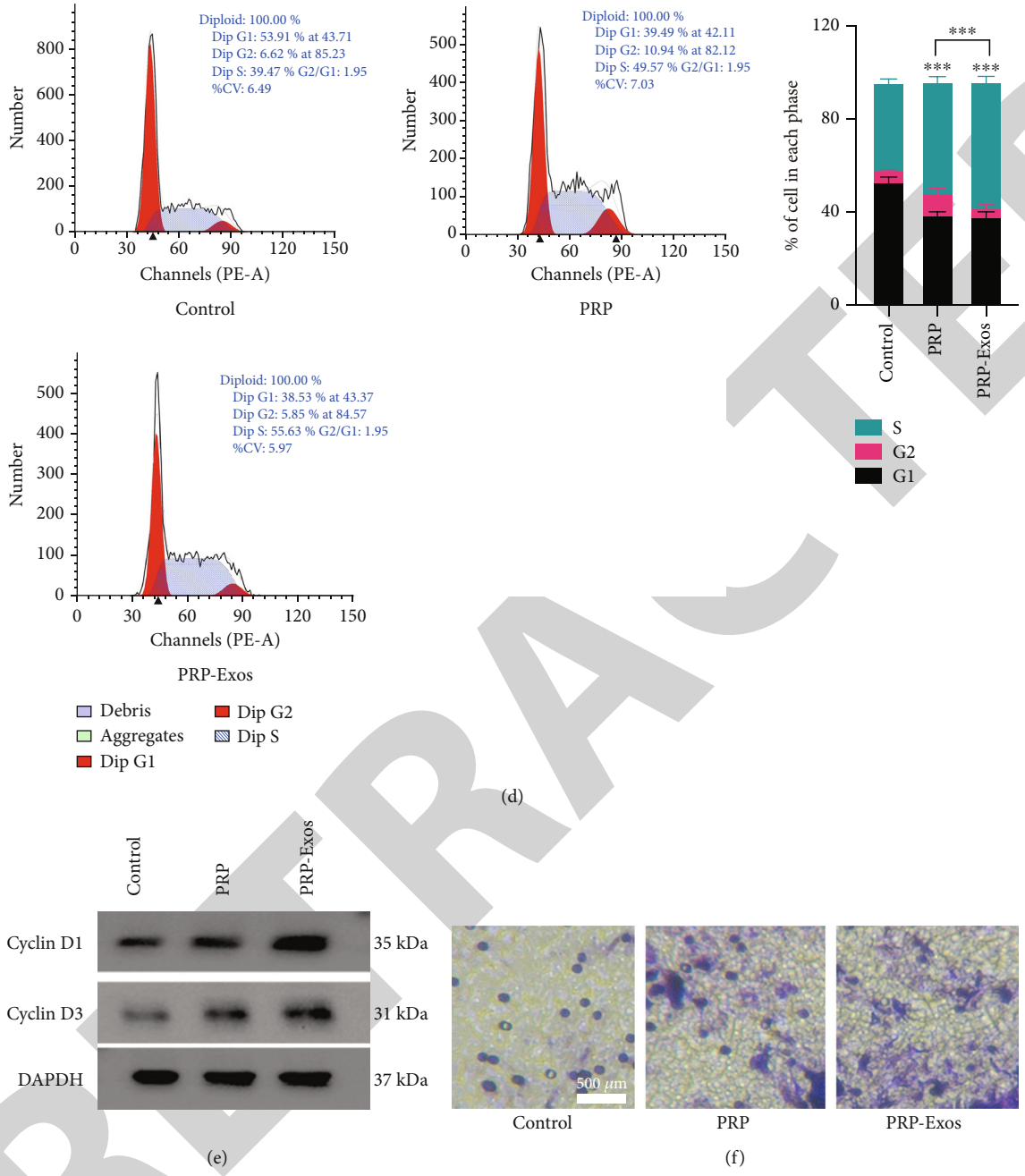

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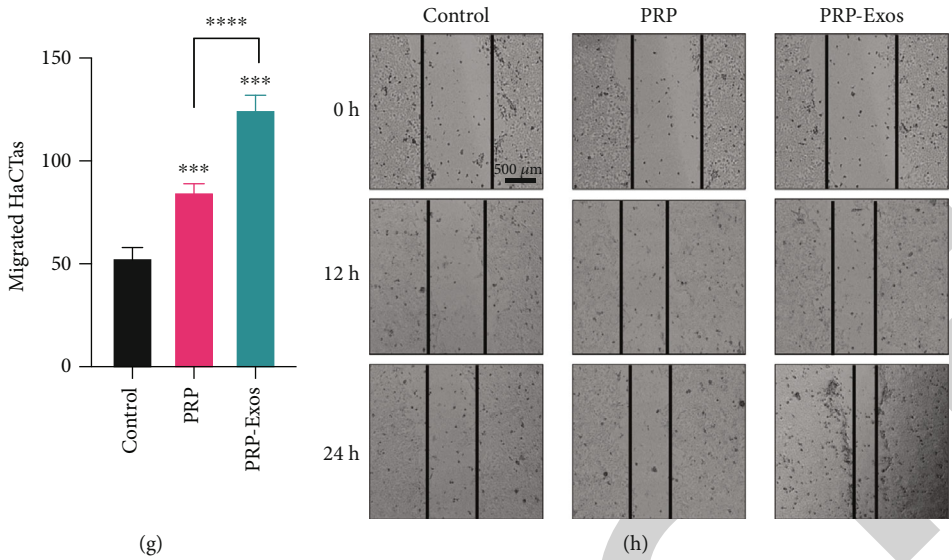

Figure 2: PRP-Exos enhance HaCaT cell proliferation and migration. (a) HaCaT cells were able to take up PKH26-labelled PRP-Exos. (b, c) CCK-8 and EdU uptake assays were used to assess the impact of PRP-Exo treatment on HaCaT cells. (d) Flow cytometry was used to assess how PRP-Exos affect cell cycle progression. (e) Western blotting was used to assess levels of cell-cycle associated proteins (cyclin D1 and cyclin D3) following PRP-Exo treatment. (f) Wound healing and (g, h) Transwell assays were used to gauge the impact of PRP-Exos on HaCaT cell migration. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

2.3. PRP-Exo Isolation and Characterization. Samples of PRP (1.5 mL) were centrifuged for 30 min at 2,000 ×*g*, after which supernatants were collected and centrifuged for 45 min at 12,000 ×*g* at 4°C, and supernatants were then passed through a 0.45 *μ*m filter membrane, after which they were centrifuged for an additional 80 min at 13,000 ×*g*. Supernatants were then discarded, while pellets were resuspended in PBS and centrifuged for 70 min at 110,000 ×*g*, and the remaining particles were suspended in chilled PBS for subsequent analysis. For transmission electron microscopy analyses, these exosomes were combined for 30 min with osmium tetroxide (4%) at 4°C in a 50 *μ*L volume, after which they were transferred onto a copper grid. Next, 1% phosphotungstic acid was utilized to stain these particles, and a transmission electron microscope (Hitachi, HT-7700) was used for their characterization. A NanoFCM™ instrument (N30E) was used for dynamic light scattering (DLS) analyses. Exosome marker protein expression was assessed via Western blotting. Exosomes collected from each subject ($n = 12$) were examined individually. Exosomes were pooled together for use in subsequent cell and animal experiments.

2.4. CCK-8 Assay. HaCaT cells were cultured in 96-well plates (5×10^3) for 24, 48, or 72 h, after which they were treated with the CCK-8 reagent for 2 h (G4103, Servicebio). Absorbance was then measured at 450 nm to assess cell proliferation.

2.5. EdU Assay. HaCaT cells were plated in 24-well plates (1×10^5/well) and treated as appropriate for 24 h, after which EdU staining was performed based on provided directions (G1601, Servicebio).

2.6. Wound Healing Assay. Following appropriate treatments, HaCaT cell monolayers cultured in 6-well plates were scratched with sterile 10 *μ*L pipette tips to generate linear scratch wounds. Cells were then incubated in the serum-free media for 12 or 24 h, after which they were imaged via inverted microscope.

2.7. Transwell Migration Assay. Cells were added to the upper chamber of a Transwell filter with an 8 *μ*m pore size (3×10^4/well) in 200 *μ*L of serum-free media, after which the lower chamber was filled with 600 *μ*L of media containing Exos or other appropriate reagents. Following incubation for 24 h, the number of migratory cells was assessed via light microscopy.

2.8. Cell Cycle Analysis. Cell cycle progression was assessed via flow cytometry using a cell cycle and apoptosis analysis kit (G1700; use G1700-50T, Servicebio) based on the provided directions.

2.9. Western Blotting. After total protein extraction, 40 *μ*g of protein per sample was separated via 10% SDS-PAGE and transferred to PVDF membranes that were stained overnight at 4°C with primary antibodies, after which they were probed for 1 h with HRP-conjugated secondary antibodies at 37°C. The following antibodies were used: anti-CD9 (1 : 1000, Abcam, ab223052), anti-TSG101 (1 : 1000, Abcam, ab125011), anti-Calnexin (1 : 1000, Abcam, ab22595), anti-USP15 (1 : 500, Abcam, ab71713), and anti-EIF4A1 (1 : 1000, Abcam, ab31217).

2.10. Quantitative Real-Time PCR (qPCR). TRIzol (Invitrogen) was used to isolate total RNA from prepared samples, after which 1 *μ*g of this RNA was used to prepare cDNA. A StepOne™ Real-Time PCR system (Life Technologies, CA, USA) was used to conduct all qPCR reactions, with the $2^{-\Delta\Delta Ct}$ method being used to assess relative gene expression and

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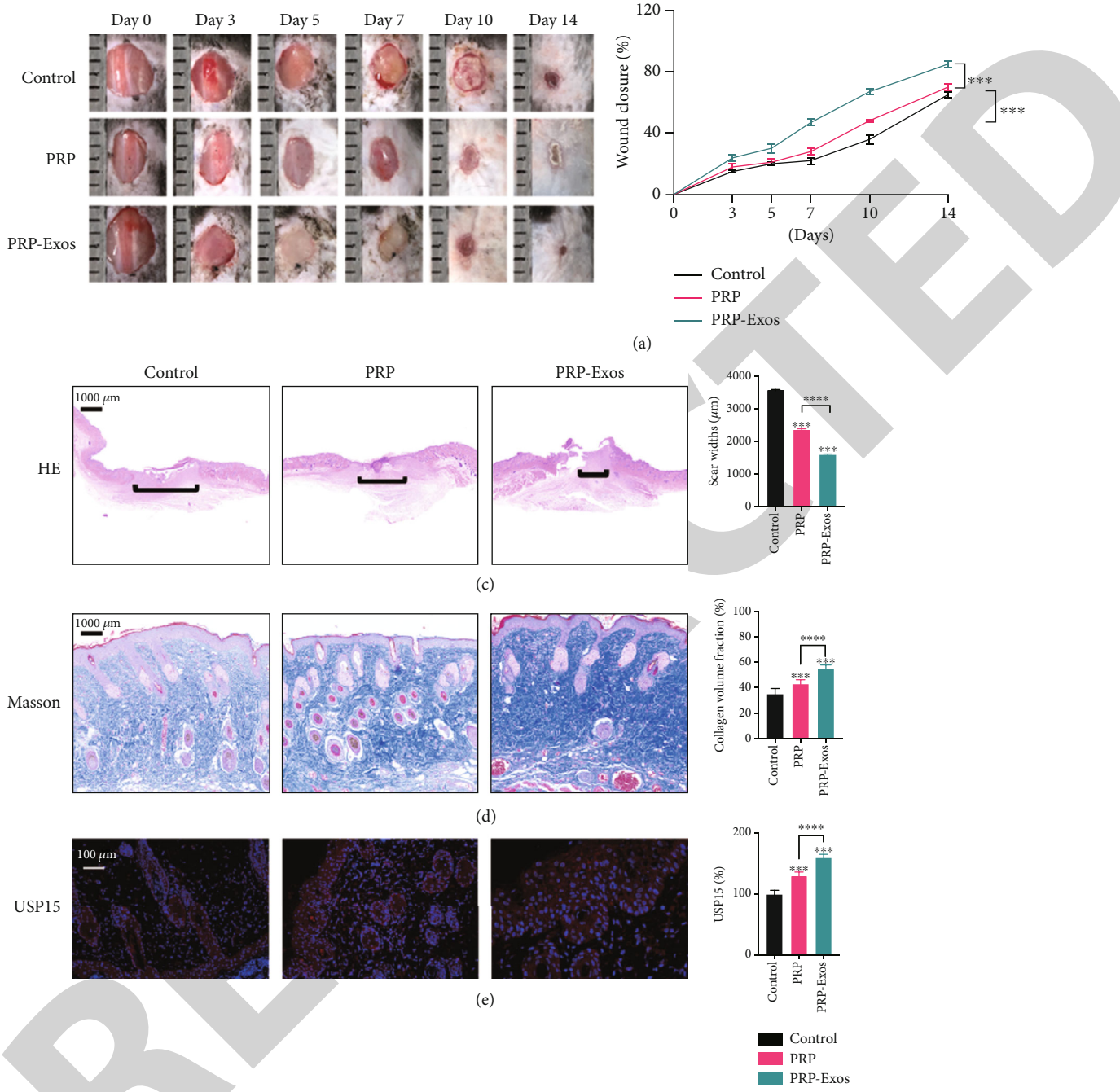

Figure 3: PRP-Exos promote wound healing in C57BL/6 mice. (a) Images of wounds in representative mice. (b) Wound healing rates in the control, PRP, and PRP-Exo treatment groups. (c, d) H&E staining and Masson's staining results from the three treatment groups with corresponding quantification results. (e) USP15 immunohistochemistry results for wound sections in the three treatment groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. $n = 6$.

GAPDH serving as a normalization control. Primer sequences were as follows: USP15: forward: 5′-AAAACCTCGCTCCG GAAAGG-3′, reverse: 5′-CCACCTTTCGTGCTATTGG-3′; EIF4A1: forward: 5′-TGTCTGCGAGCCAGGATTCCC-3′, reverse: 5′-AGATGCCACGGAGAAGGGACTC-3′.

2.11. Murine Cutaneous Wound Model Establishment. Male C57BL/6 male mice (6–8 weeks old) from the Center of Experimental Animals, Tongji Medical College, Huazhong University of Science and Technology, were individually housed in an 18°C facility with a 12 h light/dark cycle. Animals were anesthetized using intraperitoneal pentobarbital sodium with no signs of peritonitis, pain, or discomfort, after which a 10 mm diameter full-thickness excisional skin wound was generated on the dorsum of each animal. Mice were then randomly assigned to five treatment groups that were treated with PBS (100 μL), PRP-Exos (100 μg PRP-Exos in 100 μL

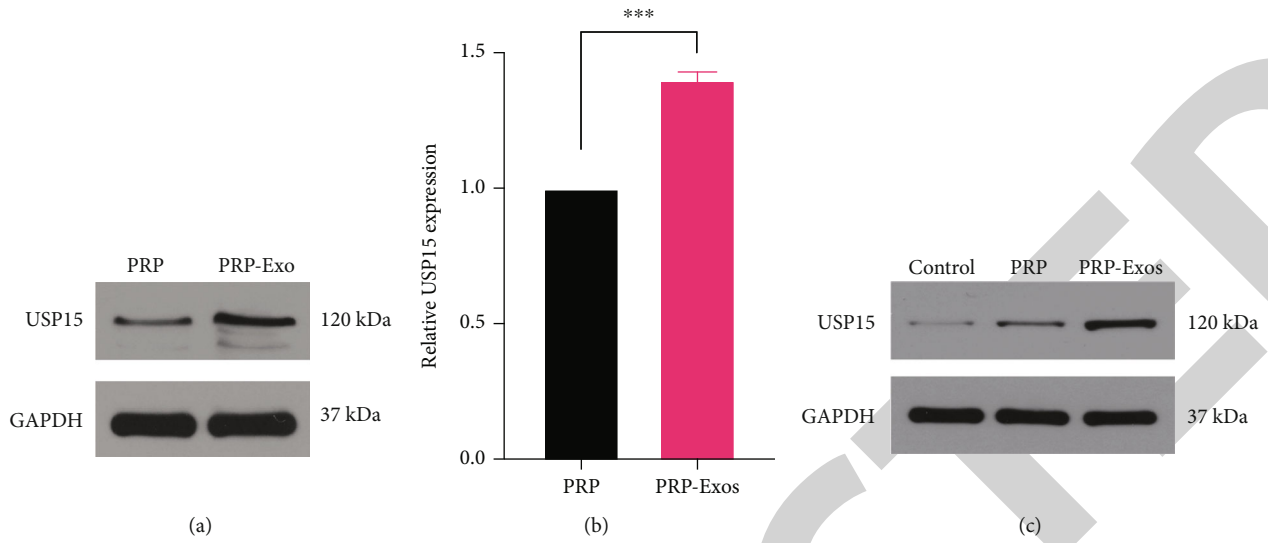

Figure 4: PRP-Exos contain high levels of USP15, which promotes enhanced HaCaT cell functionality. (a) Western blotting was used to assess USP15 levels in PRP and PRP-Exos. (b) USP15 mRNA levels were assessed via qPCR. (c) USP15 levels in murine wound tissues were assessed via Western blotting. Data are from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

PBS), siRNA-NC (in PBS), siRNA-USP15 (in PBS), or siRNA-USP15+PRP-Exos (in PBS). For siRNA-NC and siRNA-USP15 treatments, animals were administered 100 μL of a 20 μmol/L preparation of the corresponding siRNA, while for the siRNA-USP15+PRP-Exos treatment group, mice were administered 100 μL of a 10 μmol/L siRNA preparation and 10 μmol/L of PRP-Exos in PBS. All prepared solutions were injected subcutaneously in appropriate mice at four sites adjacent to the wounded area (25 μL/site). Images of the wounds were captured on days 0, 3, 7, 10, and 14 postwounding. Animals were euthanized on day 14, at which time skin samples were collected for downstream analyses. Wound area was measured with the ImageJ software, and wound healing was calculated as follows: Wound healing = (Wound area on Day n/Wound area on Day 0) (Wound area on Day n/Wound area on Day 0) × 100.

The Animal Care and Use Committee of the Tongji Medical College, Huazhong University of Science and Technology, approved all animal studies detailed herein.

2.12. Hematoxylin and Eosin Staining, Masson's Trichrome Staining, and Immunohistochemical Staining. Paraffin-embedded tissue sections (7 μm thick) were subjected to hematoxylin and eosin (H&E) and Masson's trichrome staining. An immunofluorescent approach was used to detect USP15 in prepared tissue sections. Briefly, prepared sections were blocked for 30 min with 1% BSA, probed overnight with anti-USP15 (1 : 500, Abcam, ab71713), and stained for 1 h with an appropriate secondary antibody, and then, USP15-positive cells area in three random fields of view were analyzed. All stained tissue sections were independently assessed by three observers blinded to experimental treatment protocols.

3. Results

3.1. PRP-Exo Characterization. Transmission electron microscopy (TEM), dynamic light scattering (DLS), and Western blotting were initially used to characterize isolated PRP-Exo samples. The obtained PRP-Exos ranged from 40 to 100 nm in size (Figures 1(a) and 1(b)), with the majority of these particles exhibiting cup-shaped or spheroid morphological characteristics consistent with those of Exos. Western blotting analyses indicated the presence of the exosomal markers CD9 and TSG101 in these samples (Figure 1(c)), thus confirming the successful enrichment of exosomes from PRP samples.

3.2. PRP-Exo Treatment Enhances In Vitro Keratinocyte Responses. The impact of PRP-Exo treatment on immortalized human keratinocytes (HaCaT cells) was next assessed. To establish the uptake of these exosomes by HaCaT cells, these particles were initially labeled using the lipophilic PKH26 dye, followed by incubation in cell culture media for 8 h at which time the uptake of these fluorescent particles was clearly evident (Figure 2(a)). HaCaT cells were then treated with PBS, PRP, or PRP-Exos, and their proliferation was assessed through a series of CCK-8 and EdU uptake assays, confirming that PRP-Exo exposure was associated with the enhanced proliferation of these keratinocytes (Figures 2(b) and 2(c)). PRP-Exo treatment was associated with an increase in the frequency of HaCaT cells entering the S stage of the cell cycle (Figure 2(d)). Following PRP-Exo treatment, higher levels of cell cycle-associated proteins were found to be expressed in these cells (Figure 2(e)). In Transwell and wound healing assays, PRP-Exo treatment was also associated with significant improvements in HaCaT cell migration (Figures 2(f)–2(h)). Together, these data thus indicate that PRP-Exo treatment can significantly augment HaCaT cell proliferation and migration.

3.3. PRP-Exo Treatment Enhances In Vivo Wound Healing in Mice. An *in vivo* cutaneous wound model was next established using C57BL/6 mice, with equal volumes of PBS, PRP, or PRP-Exo preparations being injected around the

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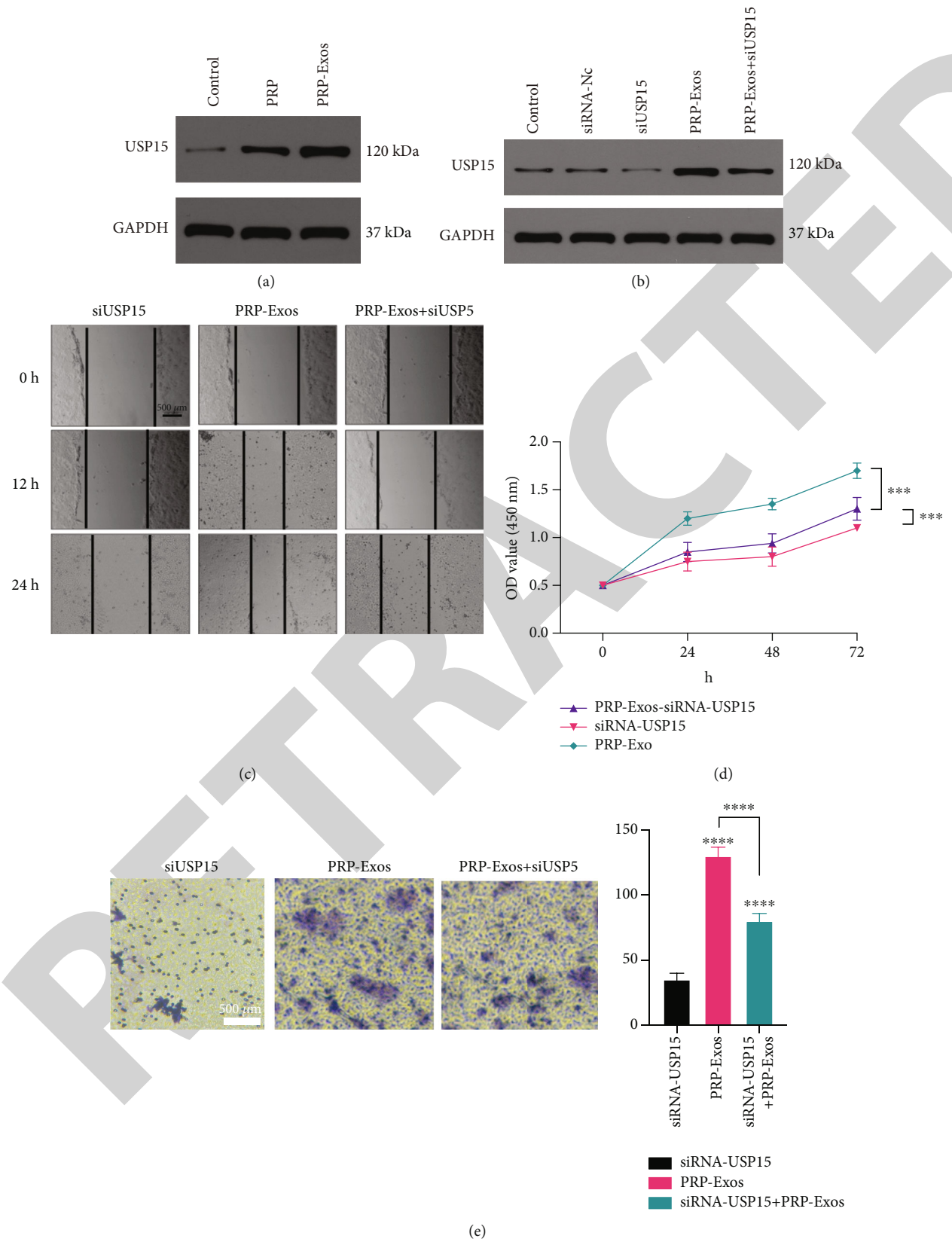

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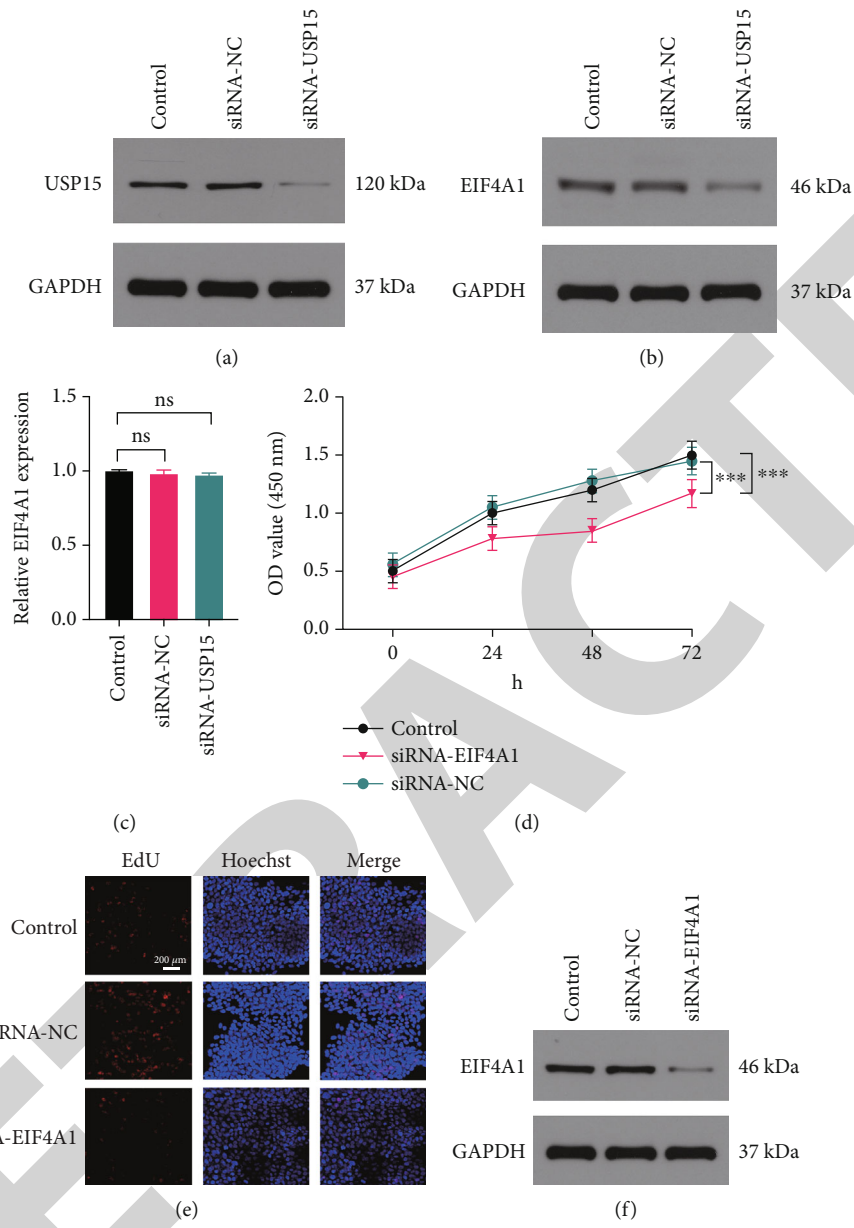
Figure 5: USP15 promotes in vitro wound healing. (a) Western blotting data demonstrating USP15 levels in control, PRP, and PRP-Exos groups. (c, e) Wound healing and Transwell assays were used to assess the migratory activity of HaCaT cells. (d) The proliferation of HaCaT cells in the indicated treatment groups was assessed via CCK-8 assay. Data are from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



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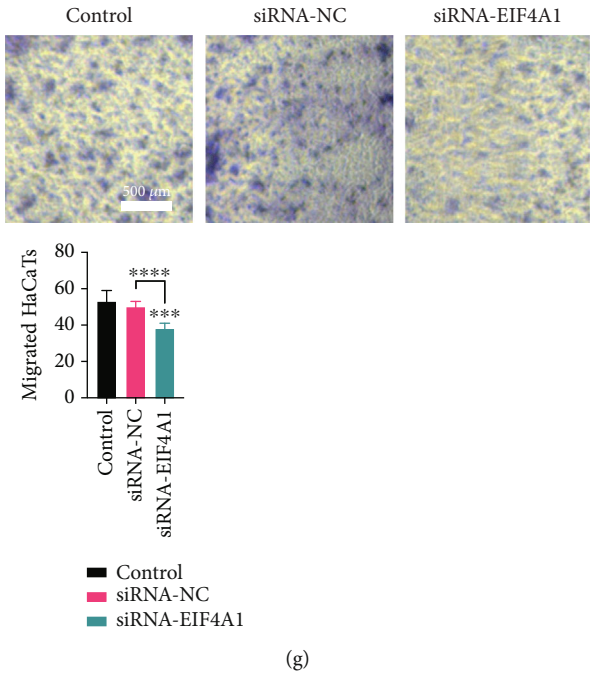

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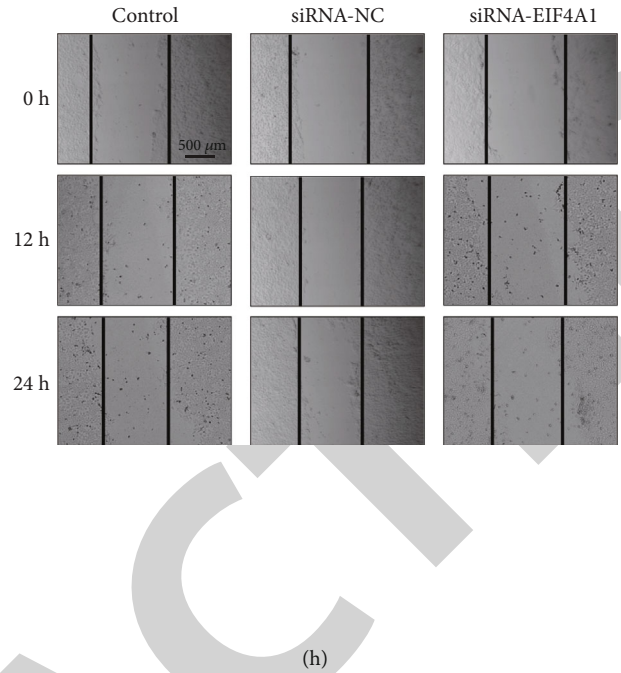

(h)

Figure 6: USP15 promotes EIF4A1 deubiquitination to enhance HaCaT cell functionality. (a, b) Western blotting results demonstrating USP15 and EIF4A1 levels in HaCaT cells following siRNA-USP15 treatment. (c) EIF4A1 levels in HaCaT cells following siRNA-USP15 treatment, as assessed via qPCR. (d, e) HaCaT cell proliferation was assessed via CCK-8 and EdU assays following siRNA-EIF4A1 treatment. (f) Western blotting analyses were used to assess EIF4A1 expression HaCaT cells following siRNA-EIF4A1 treatment. (g, h) Wound healing and Transwell assays were used to assess the migratory activity of HaCaT cells following siRNA-EIF4A1 treatment. Data are from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

wound site in each animal to assess the impact of such treatment on wound healing. Relative to control animals, those treated with PRP and PRP-Exos exhibited faster wound repair, with such healing being even more rapid for PRP-Exo-treated mice relative to mice treated with PRP alone (Figures 3(a) and 3(b)). The scars of mice in the PRP-Exo group were smaller than those of mice in any other group (Figure 3(c)), and these mice exhibited the highest levels of collagen formation (Figure 3(d)). Reepithelialization plays an essential role in the wound healing process [16]. Deubiquitinases (DUBs) can alter protein stability by removing a ubiquitin chain from a given protein, thereby stabilizing it [17], thus potentially accelerating the process of reepithelialization in the context of wound healing. The ubiquitin-specific protease (USP) family is the best-studied group of DUB proteins, and USP15 is an important member of this family [18]. As such, we next evaluated the expression of USP15 in keratinocytes in wounded skin tissues, revealing it to be expressed at higher levels in the PRP-Exo treatment group (Figure 3(e)).

3.4. USP15 Is Enriched in PRP-Exo Preparations. To explore the functional importance of USP15 as a driver of PRP-Exo-mediated wound healing, we assessed the levels of this protein in PRP and PRP-Exo samples via Western blotting, revealing it to be present at significantly higher levels in PRP-Exos (Figure 4(a)). Subsequent qPCR analyses additionally exhibited increased USP15 expression in PRP-Exos at the mRNA level (Figure 4(b)). Similarly, higher USP15 protein levels were evident in epidermal keratinocytes in the PRP-Exo group *in vivo* (Figures 4(c) and Figure 3(e)), suggesting that USP15 may be linked to the ability of PRP-Exos to promote wound healing.

3.5. PRP-Exos Promotes HaCaT Cell Migration and Proliferation in a USP15-Dependent Manner. To understand the functional effects of USP15 on HaCaT cells, they were next treated with PBS, PRP, and PRP-Exos. Western blotting indicated that USP15 levels were highest for HaCaT cells treated with PRP-Exos (Figure 5(a)). As shown in Figure 1, HaCaTs treated with PRP-Exos exhibited significantly augmented cellular proliferation and migration. In contrast, siUSP15 treatment had the opposite effect on HaCaT cell proliferation and migration in these same assay systems (Figures 5(b)–5(e)). As such, decreasing USP15 expression in HaCaT cells can reduce the beneficial impact of PRP-Exo treatment. Together, these findings suggest that USP15 is the primary mediator whereby PRP-Exos promote HaCaT cell migration and proliferation.

3.6. USP15 Enhances HaCaT Cell Functionality by Promoting EIF4A1 Deubiquitination. We next explored the mechanisms whereby USP15 promotes HaCaT cell functionality. EIF4A1 is a key eukaryotic initiation factor complex component that has been linked to the proliferation of certain cell lines [19, 20], although it has not been studied in detail in HaCaT cells. As such, we hypothesized that USP15 may be able to promote HaCaT cell proliferation in part via altering the

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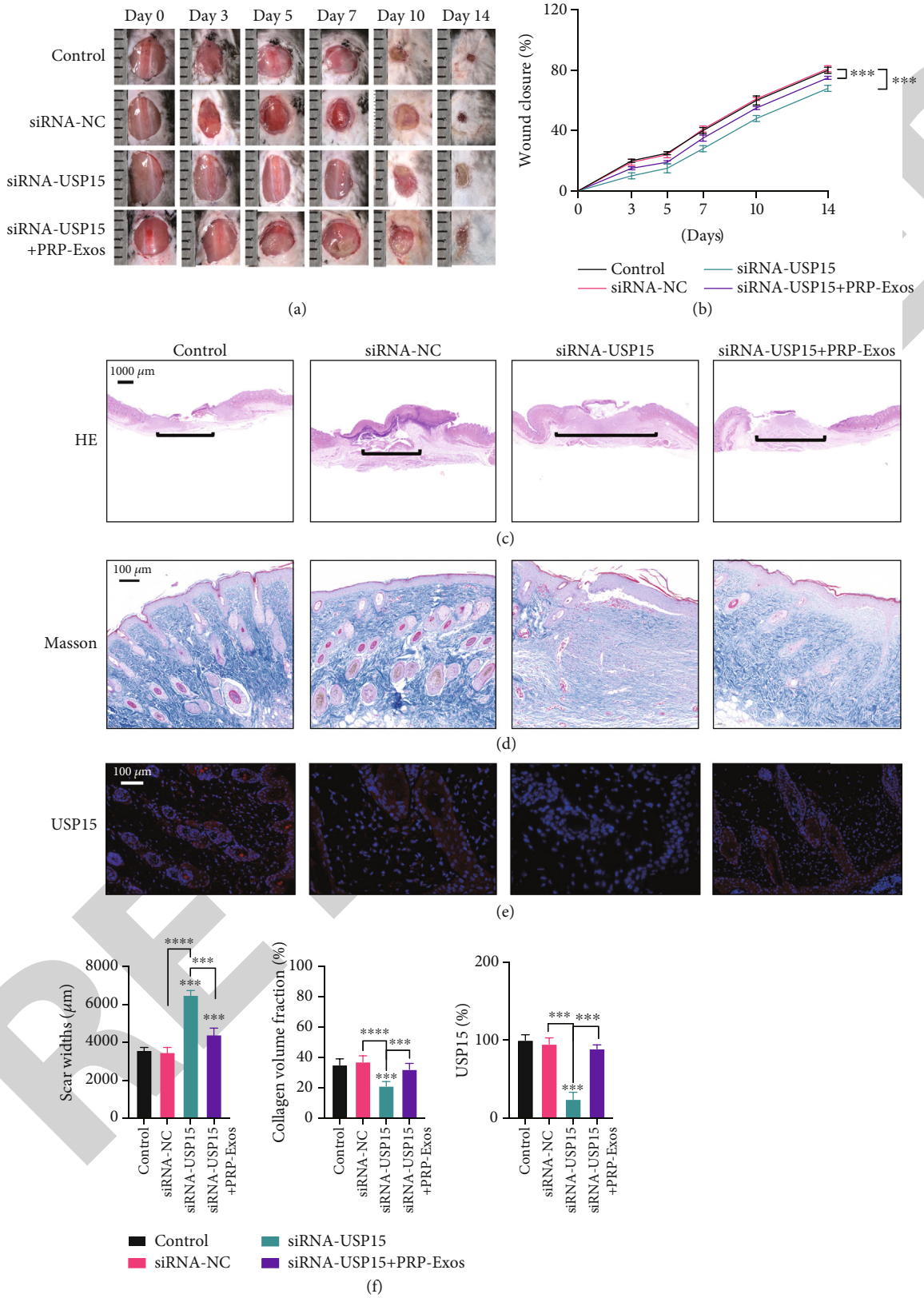

Figure 7: USP15 promotes in vivo wound healing. (a) Images of mice in the three treatment groups. (b) Wound closure rates for mice in the three treatment groups. (c) H&E staining results from the three treatment groups. (d) Masson's trichrome staining results for wounds in the three treatment groups. (e) USP15 immunohistochemical staining results for the three groups. (f) Quantification results for data in the three treatment groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. $n = 6$.

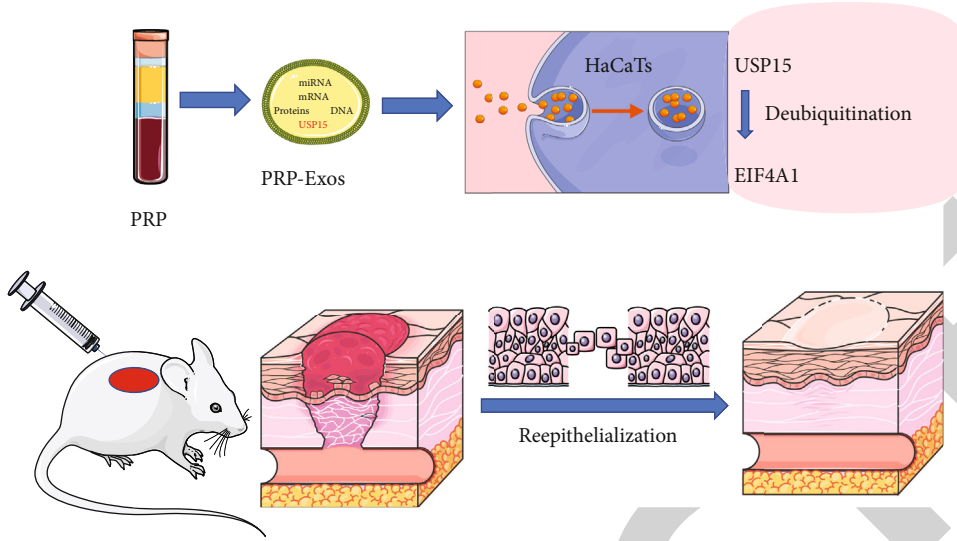

Figure 8: USP15 is one of the main mRNA in PRP-Exos, which could be taken into HaCaTs, subsequently deubiquitinating EIF4A1, then accelerating reepithelialization and promoting wound healing.

EIF4A1 expression. To examine the effects of USP15 on EIF4A1 stability in HaCaT cells, we conducted Western blotting assays which revealed a significant decrease in both USP15 and EIF4A1 expressions following siRNA-USP15 treatment (Figure 6(a) and 6(b)), whereas qPCR analyses indicated that the EIF4A1 mRNA levels in these cells were unchanged (Figure 6(c)).

We then assessed the functional importance of EIF4A1 in HaCaT cells by knocking down this gene with a specific siRNA construct. Subsequent CCK-8 and EdU assays suggested that EIF4A1 knockdown was sufficient to suppress HaCaT cell proliferation (Figures 6(d) and 6(e)), with Western blotting being used to confirm that EIF4A1 protein levels were reduced following siRNA transfection (Figure 6(f)). In Transwell and wound healing assays, siRNA EIF4A1 treatment significantly reduced the migratory activity of these keratinocytes (Figures 6(g) and 6(h)). Together, these data suggested that USP15 can enhance the migration and proliferation of HaCaT cells by promoting EIF4A1 deubiquitination.

3.7. USP15 Promotes Wound Healing In Vivo. To examine the impact of USP15 on wound healing processes, equivalent amounts of PBS, siRNA-NC, siRNA-USP15, siRNA-USP15, or PRP-Exos were injected surrounding wound sites in C57/BL6 mice, as above. USP15 knockdown was found to significantly slow the wound healing process in these animals (Figures 7(a) and 7(b)), and H&E staining confirmed that wounds in the siRNA-USP15 group were larger than those in other groups (Figure 7(c)). Masson's trichrome staining also revealed that collagen levels were lowest in the siRNA-USP15 group (Figure 7(d)), and the number of USP15-positive cells in murine wound sites was lower in siRNA-USP15-treated mice relative to other groups (Figures 7(e) and 7(f)). Together, these results thus demonstrated that USP15 knockdown was sufficient to largely ablate the beneficial effects of PRP-Exo treatment in the context of *in vivo* cutaneous wound healing.

4. Discussion

A number of clinical strategies have been employed in recent years in an effort to accelerate wound healing [21]. PRP-based therapies have emerged as promising means of promoting wound healing and have been studied in the fields of orthopedics, dermatology, dentistry, and diabetic wound management. However, there have been few studies to date of PRP-Exos. Herein, we determined that PRP-Exo treatment was sufficient to promote wound healing in a manner more efficient than direct PRP treatment. The process of epidermal regeneration is a multistep process that is regulated by a range of cytokine and cell types, ultimately leading to the reconstruction of the damaged skin barrier [22]. Exosomes derived from mesenchymal stem cells have been repeatedly shown to accelerate reepithelialization and to thereby enhance wound healing [23, 24]. Our present results indicated that PRP-Exo treatment similarly promoted enhanced epithelialization, highlighting the promise of these particles for use in the treatment of chronic wounds.

Exosomes are small, lipid bilayer-enclosed extracellular vesicles that are produced by most known cell types and that can transmit macromolecules and other compounds between cells [25]. When taken up by recipient cells, these exosomes can thus alter cellular functionality via the delivery of specific proteins, nucleic acids, and signaling molecules in a manner that makes them ideal for use as drug carriers in a range of disease types [24]. Herein, we found that HaCaT cells were able to efficiently internalize PRP-Exos. It has been reported that a range of new types of hydrogels with specifically tailored biochemical functions can accelerate wound healing, thus exhibiting good prospects for therapeutic application [26, 27]. However, such artificial hydrogels play a less direct role in normal physiological processes, and a combination of exosomes and hydrogels may thus offer greater advantages further expediting the wound healing process.

Protein ubiquitination has been shown to play diverse regulatory roles in the context of wound healing [28, 29].

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USP family proteins, including USP15, are key mediators of protein deubiquitination. Others have reported that PRP can promote wound healing by driving accelerated epithelialization [30], and we herein found that PRP-Exo treatment was superior to PRP treatment as a means of enhancing wound healing in a manner associated with increased USP15 protein levels in wound tissues relative to those detected upon PBS or PRP administration. Through a series of *in vitro* and *in vivo* experiments, we further confirmed that USP15 was able to promote wound healing, suggesting that PRP-Exo-derived USP15 is a key mechanism driving this regenerative process. Tao et al. have reported that PRP-Exos can suppress apoptosis in a rat model of femoral head osteonecrosis via the Akt/Bad/Bcl-2 signaling pathway [31], while Iyer et al. revealed the ability of PRP-Exo treatment to promote functional recovery following muscle injury [32]. Owing to their unique properties, exosomes have been shown to be of value for the treatment of many diseases and thus warrant further clinical study.

In prior studies, USP15 was shown to interact with EIF4A1 and to thereby accelerate wound healing [33]. Consistent with such a model, we found that USP15 knockdown was sufficient to reduce EIF4A1 expression, while EIF4A1 knockdown directly impaired HaCaT cell migration and proliferation. Together, these results suggest that the USP15-EIF4A1 axis is a key mediator of the reepithelialization process.

5. Conclusion

In conclusion, the results of this study indicate that PRP-Exo-derived USP15 is a key mediator of HaCaT cell survival and migratory activity, with EIF4A1 playing an important role in the process of USP15-induced epithelialization (Figure 8). Together, these findings provide a robust foundation for future studies of the therapeutic potential of PRP-Exo treatment as a means of promoting improved wound healing.

Data Availability

Data available on request.

Conflicts of Interest

All authors report no conflicts of interest in this work.

Authors' Contributions

Yan Xu and Ze Lin contributed equally to this work and should be regarded as co-first authors.

Acknowledgments

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Review Article

Role of Exosomal MicroRNAs and Their Crosstalk with Oxidative Stress in the Pathogenesis of Osteoporosis

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Osteoporosis (OP) is an aging-related disease involving permanent bone tissue atrophy. Most patients with OP show high levels of oxidative stress (OS), which destroys the microstructure of bone tissue and promotes disease progression. Exosomes (exos) help in the delivery of microRNAs (miRNAs) and allow intercellular communication. In OP, exosomal miRNAs modulate several physiological processes, including the OS response. In the present review, we aim to describe how exosomal miRNAs and OS contribute to OP. We first summarize the relationship of OS with OP and then detail the features of exos along with the functions of exo-related miRNAs. Further, we explore the interplay between exosomal miRNAs and OS in OP and summarize the functional role of exos in OP. Finally, we identify the advantages of exo-based miRNA delivery in treatment strategies for OP. Our review seeks to improve the current understanding of the mechanism underlying OP pathogenesis and lay the foundation for the development of novel theranostic approaches for OP.

1. Introduction

Osteoporosis (OP) is a disease caused by abnormal bone metabolism [1] due to the dysfunction and abnormal differentiation of osteoclasts and osteoblasts [2, 3]. Patients with OP show increased osteoclast differentiation and decreased osteoblast differentiation [3]. The loss of bone density in OP weakens bone structure, leading to fractures. OP can also result in pain and other complications, reducing a patient's ability to engage in activities of daily living. The mechanism underlying OP remains unclear, although recent studies have been successful in exploring its biology in further detail [4].

Oxidative stress (OS), a risk factor for OP, has received increasing attention in recent years. Studies have shown that OS can inhibit the differentiation of osteoblasts in bone marrow and stimulate the differentiation of osteoclasts, promoting the occurrence and development of OP [5, 6]. OS occurs as a result of the constant production of reactive oxygen species (ROS), such as superoxide anions (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and other free radicals, during metabolic processes in the human body [7]. Superoxide dismutase, glutathione peroxidase, catalase, and reduced glutathione constitute the main antioxidant defense systems in humans [8]. In addition, dietary antioxidants can complement the endogenous antioxidant system [9].

Under normal circumstances, there is a balance between ROS production and the body's antioxidant defense [10]. Controlled production of free radicals by normal osteoclasts can accelerate the destruction of calcified tissue and contribute to bone reconstruction [11, 12], which is very important for the growth and development of bones and for fracture repair. However, factors such as smoking, aging, and estrogen deficiency can disrupt the redox balance, resulting in OS [13]. OS causes extensive oxidative damage to cells—it inhibits the differentiation, growth, and proliferation of most cells and accelerates cell aging and death via the activation of several signal transduction pathways, such as the NF-κB, MAPKs, p53, and HSF pathways [14, 15].

Therefore, OS also affects bone remodeling.

Bone remodeling is coordinated and regulated by osteoclasts and osteoblasts, and studies suggest that these two cell types communicate with each other [16, 17]. In addition to

substantial evidence demonstrating that osteoblasts guide osteoclast bone resorption, studies have also shown that osteoclasts regulate osteoblast bone formation through direct cell-to-cell contact and cytokine-mediated indirect contact [18]. However, it is unclear whether any other "paracrine" pathways mediate the communication between osteoclasts and osteoblasts [19].

Recently, exosomal miRNAs have been shown to regulate several physiological processes, including the OS response, in OP [20–23]. Our review seeks to enhance the understanding of the mechanism underlying OP pathogenesis and to lay the foundation for novel theranostic approaches for OP.

2. OS and OP

OS is caused by the build-up of free radicals, including those generated as a result of inflammation and mitochondrial dysfunction [24]. ROS are the primary contributors to the aggravation of OS and tissue damage [25]. ROS production and clearance are a dynamic process that is affected by multiple factors. Under normal conditions, appropriate levels of ROS are required to maintain certain signaling pathways, enhance cell proliferation, and regulate cell metabolism [26]. However, when the normal redox state of cells is disturbed, peroxides and free radicals are produced. This causes damage to all cell components, including proteins, lipids, and DNA, leading to cellular toxicity [7, 8, 27].

OP is a systemic skeletal disorder [28, 29] that results from reduced maximum bone mass levels and elevated bone loss [30]. Given the involvement of abnormal metabolism in OP, this disease is particularly sensitive to OS, and the relationship between the two has therefore gathered significant attention. Moreover, OS is known to contribute to diseases of bone metabolism, especially OP, as elevated OS is often observed in the bone tissue of OP patients. Therefore, OS may be a potential target for the treatment of OP [31, 32].

Previous findings demonstrate the detrimental effect of OS on bone health [33–35]. ROS are thought to affect the bone environment via two modes of action. Primarily, ROS may potentiate the responsiveness of osteoclast precursors to RANKL, and secondarily, it may induce the production of additional osteoclastogenic cytokines such as IL-1, IL-6, and IL-7 [36, 37]. Furthermore, OS may also affect the function of osteoblasts. Recent studies have shown that ROS decrease the life span of osteoblasts in osteoporotic mice [38]. Interestingly, both endogenous and dietary antioxidants were found to mitigate and delay bone loss in a number of animal studies. Moreover, various forms of vitamin E have been found to prevent the reduction in trabecular number and bone volume in osteoporotic mice [39, 40]. In addition, a number of epidemiological studies have demonstrated that bone mass density has a positive and inverse relationship with OS biomarkers and antioxidant status, respectively.

3. Exosomes (Exos) and Exo-Associated miRNAs

3.1. Biological Characteristics of Exos. Exos are extracellular vesicle-like substances with a diameter of 30–150 nm and are found in almost all functional and nonfunctional biological fluids. Exos are a part of a large family of membrane vesicles, which also includes extracellular microvesicles (100–350 nm) [41] and apoptotic vesicles (500–1000 nm) [42, 43]. Exos are thought to be involved in many biological processes and play an important role in cell-to-cell communication [44–46]. Most cells release exos into the extracellular environment after plasma membrane fusion [47–49]. The discovery of exos dates back to 1983, when researchers cultured reticulocytes to track the movement of transferrin receptors from the plasma membrane to reticulocytes. Surprisingly, they found that the tagged transferrin receptors were taken up by reticulocytes and then reassembled into small vesicles within reticulocytes. At first, it was thought that these vesicles would be destroyed by lysosomes inside the cell and then expelled out of mature red blood cells, but the actual functions of these vesicles were subsequently discovered.

Lipids and proteins are the main active constituents of exos, and a variety of nucleic acids, including mRNA, miRNAs, and other noncoding RNAs, have also recently been found to be present [50]. When exos are secreted and released into body fluids, they can reach target cells. After being taken up by target cells, exos can release active RNAs and therefore play a role in the subsequent regulatory processes (Figure 1).

The mechanism underlying the identification and internalization of exos is a key focus of investigation. According to evidence from recent studies, exo uptake is specific. Moreover, exos are adept at delivering their contents to specific acceptor cells. For instance, exos released by fibroblasts (NIH-3T3 cells) are capable of delivering antagomir-188 to mesenchymal stem cells (MSCs) in a bone-targeted manner [51]. Moreover, MSC-derived exos can promote angiogenesis and osteogenesis by delivering exosomal miR-29a [52]. However, the main pathway governing the delivery of exos to specific target cells remains unclear, although there are some hypotheses to explain this phenomenon. One hypothesis suggests that target cells identify and engulf exos based on their size and membrane components [53, 54]. For example, CD47 on the exosomal membrane may prevent the endocytosis of exos by monocytes and macrophage [55]. Additionally, CD11a and CD54—which are present on the surface of dendritic cells—and CD9 and CD81—which are present on the surface of exos—may promote exosomal targeting to dendritic cells as well as their engulfment [56]. Another hypothesis suggests that the molecular cargo carried by exos itself targets exos to specific cells, but evidence supporting this postulation is lacking. Given the extensive potential of exos in targeted therapy, the molecular and cellular mechanism via which they maintain their specificity warrants additional investigation.

3.2. Exosomal miRNAs

3.2.1. Characteristics. miRNAs are small noncoding RNAs (17–24 in length) that bind to the 3′UTR or open reading frame of target mRNAs and regulate posttranscriptional gene silencing [57]. The miRNAs present in exos can be delivered to neighboring or distant cells, where they exert regulatory

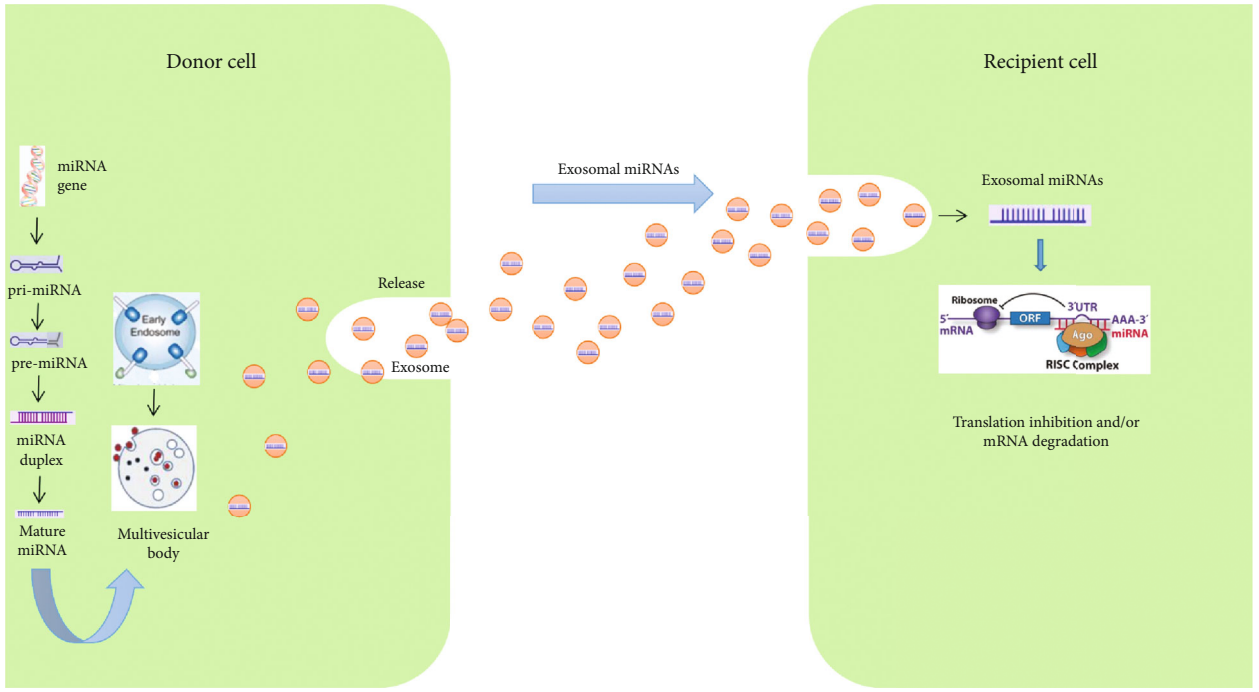

Figure 1: Exosomal miRNA biogenesis and interaction with target cells. A variety of miRNAs are contained in exos and delivered to target cells.

effects. Exosomal miRNAs play a key role in bone metabolism-related disease progression [20, 58, 59], and three potential mechanisms have been implicated in their pathogenic role. First, miRNAs are thought to target mRNAs of regulatory genes and suppress their translation or promote protein degradation. Second, miRNAs are thought to contribute to OP pathogenesis by directly binding to toll-like receptors or regulating their transcriptional expression. Finally, miRNAs are considered to cause a miRNA formation disorder [60]. Moreover, the current evidence indicates a strong relationship between miRNAs and OS.

3.2.2. Role of Exosomal miRNAs in OP

(1) Novel Avenues for Gene Therapy. Exos can deliver functional miRNAs and have thus been used to develop exo-based targeted gene therapy [61]. Owing to their high biosafety and immune evasion abilities, exos have great potential as miRNA vectors [62]. Currently, exo-based miRNA delivery systems are being explored using animal models [63]. Duan et al. confirmed the efficacy of miR-140 delivery using engineered exos for osteoarthritis therapy [64]. The expression of miRNAs changes across the different phases of OP, and some miRNAs play a role in OP progression [65]. Consequently, the control of miRNA expression using exos could be a feasible approach for gene therapy in cases of OP.

(2) Exo-Based Cell–Cell Communication. Chemical receptor-mediated communication is the most well-documented form of cell–cell communication [66]. Exos and their transport across different cells have widened our understanding of cell–cell communication. miRNAs, one of the most important elements present in exos, have been demonstrated to participate in cell–cell communication [67]. For an example, the circulating exosomal miR-20b-5p that is released from cells in patients with diabetic foot ulcers is known to transmit functional information via paracrine secretion and regulate diabetic wound healing—a process dependent on various cells, including vascular endothelial cells and fibroblasts [68]. Furthermore, M2 macrophages can deliver miR-5106-containing exos to bone marrow MSCs (BMSCs) and regulate protein expression in these cells [69]. Given the importance of BMSCs in bone remodeling, their role in the onset and progression of OP is unsurprising. Taken together, this evidence may provide novel avenues for examining the association of exosomal miRNAs with OP.

4. Crosstalk between Exosomal miRNAs and OS in OP

Both OS and exo-derived miRNAs play crucial roles in the occurrence of OP. Interestingly, OS regulates many miRNAs, and conversely, miRNAs also regulate genes participating in the OS response [70]. A recent study found that OS upregulates the expression of miR-34a in exosomes derived from muscles, and this miRNA then induces cellular senescence in bone stem cells. In C2C12 myoblasts, the overexpression of exosomal miR-34a suppresses Sirt1 mRNA and protein expression [71]. miR-34a induces senescence in vascular smooth muscle cells and cardiomyocytes and promotes cardiac fibrosis [72, 73]. It has been suggested that with aging and increased exposure to inflammatory factors and ROS, both of which increase OS, miR-34a is upregulated as a

Table 1: The role of exosomal miRNAs in osteoporosis.

Exosome source	RNA extraction	RNA identification	Exosomal miRNA(s)	Regulatory role	Reference
Human bone marrow mesenchymal stem cells	Exosome extraction kits (QIAGEN, Germany)	RT-qPCR	miR-186	Ameliorate	Li et al., 2021, [81]
Human umbilical cord mesenchymal stem cell	Trizol reagent	RT-qPCR	miR-1263	Ameliorate	Yang et al., 2020, [80]
Vascular endothelial cell	Trizol reagent	RT-PCR	miR-155	Ameliorate	Song et al. 2019, [79]
Bone marrow stromal cells of aging mice	Trizol reagent	RT-qPCR	miR-31a-5p	Aggravate	Xu et al., 2017, [78]
Bone marrow mesenchymal stem cells	Trizol reagent	RT-qPCR	miR-29a	Ameliorate	Lu et al., 2020

consequence of p53 activation, which occurs in cases of sepsis, injury, and inflammation [74].

miR-182-5p was previously reported to inhibit osteoblast proliferation and differentiation by targeting Foxo1 [75], and miR-183-5p was found to be elevated during cellular senescence after exposure to OS [76]. Davis et al. found that bone-derived exos are capable of impairing MSC proliferation and inducing bone stem cell senescence. Moreover, miR-183-5p, an exosomal miRNA, was demonstrated to be a significant active contributor to this regulation. Furthermore, *in vitro* assays based on H_2O_2-induced OS have indicated that H_2O_2 treatment increases the abundance of miR-183-5p in bone-derived exos in MSCs and that H_2O_2 levels in the bone marrow microenvironment increase with age [76].

5. Role of Exosomal miRNAs in OP

Owing to the biological characteristic of exos, exosomal miRNAs can exist stably in the body and can remain stable for 48 hours at 4°C *in vitro* [77]. These special features allow exos to play a regulatory role in certain diseases. The functional effects of exosomal miRNAs in OP have been well documented (Table 1) [52, 78–81]. Li et al. reported that exos derived from MSCs can effectively ameliorate the development of OP, and exosomal miR-186 participates in this regulatory process [81]. Moreover, a recent study demonstrated that exosomal miR-1263 derived from human umbilical cord MSCs can inhibit osteoblast apoptosis and that nanomaterials loaded with miR-1263 may be ideal alternatives for the treatment of bone resorption disorders [80]. Song et al. assessed upregulated miR-155 levels in exos derived from vascular endothelial cells and suggested that the exos and the exosomal miR-155 may serve as bone-targeting and nontoxic nanomedicines for the treatment of OP [79]. Furthermore, Xu et al. reported that miR-31a-5p levels are significantly elevated in exos from aging BMSCs. These levels contribute to age-related changes in the bone marrow microenvironment and affect osteoblastic and osteoclastic differentiation [78]. In addition, exosomal miR-29a was recently demonstrated to inhibit OP progression by promoting osteogenesis and angiogenesis [52]. Therefore, exosomal miRNAs play important roles in the development of OP.

6. Exo-Based miRNA Delivery for OP Treatment

The blood–brain barrier (BBB) is one of the most challenging hindrances against treatment strategies for bone remodeling diseases, delaying the development of novel clinical agents [82]. Exos can traverse the BBB, and studies have reported that exos have many additional advantages as delivery vehicles for drugs and nanoparticles [83], including a high delivery efficiency, good biocompatibility, and efficient management of the inflammatory response [84]. The exo-mediated delivery of miRNAs for OP treatment has become the focus of recent research. MSC-derived exos have been found to be effective in delivering functional miRNAs that promote osteogenic differentiation and inhibit the development of OP [78–80]. Recent studies have demonstrated that local or systemic application of exos has potential as a treatment option for OP [85–88]. Current research on exos has advanced beyond the observation stage, and convincing experimental results have been obtained. However, from the perspective of clinical applications, these results should be interpreted with caution. First, there is currently no widely applicable method for exo isolation and validation. Existing isolation techniques lead to the inevitable mixing of nonexosomal components, such as lipoproteins, proteins, viruses, and bacteria, with exos isolated from different specimens [89–91]. In addition, the standards for separation are not uniform, and the different equipment used across different laboratories may lead to further differences and inconsistencies, which will eventually lead to different findings [92, 93]. The techniques used for the characterization of exos are also different, and their accuracy varies too [94]. Finally, a variety of methods are used to determine the concentration of exos, including simple quantitative protein determination and nanoparticle tracking analysis, and a wide variety of units are used for quantitation [95, 96].

Owing to these problems, it is necessary to standardize sample collection methods and methods for separation, characterization, and quantitation in order to facilitate the collection of reliable and replicable data across different laboratories and research areas. Furthermore, researchers need to be aware of the challenges involved in the experimental procedures put forth in the recent guidelines from the International Society of Extracellular Vesicles. Given that a gold standard for exo isolation and characterization has not

been established, researchers should perform thorough literature reviews to identify the most suitable isolation method for their research.

7. Conclusion and Perspectives

In summary, exosomal miRNA-mediated OS affects osteoblasts, osteoclasts, and the bone matrix, promoting the development of OP. Antioxidants have the potential to inhibit OS. When the antioxidant balance in the body is disrupted, exogenous antioxidants can help in preventing or postponing the development of OP. However, current research in this field is limited. Therefore, it is necessary to conduct in-depth basic and clinical studies to clarify the role of OS and exosomal miRNAs in the occurrence and development of OP and to develop novel and improved treatments for this disease.

Data Availability

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

Conflicts of Interest

All the authors declare that there are no conflicts of interest.

Authors' Contributions

Jun Lu and Yan Zhang contributed equally to this work.

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