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Bone marrow stem cells derived exosomes improve osteoporosis by promoting osteoblast proliferation and inhibiting cell apoptosis

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether bone marrow stem cells (MSCs) derived exosomes in rats could promote osteoblast proliferation and improve osteoporosis via inhibiting cell apoptosis.

MATERIALS AND METHODS: MSCs in rats were isolated and cultured, followed by the identification of surface antigens *via* flow cytometry. The differentiation of MSCs was detected by alizarin red staining and oil red staining. After extraction from MSCs by ultracentrifugation, the size distribution of exosomes was detected by tunable resistive pulse sensing (TRPS). Specific antigens in MSCs-derived exosomes were determined by flow cytometry. Furthermore, the proliferation and viability of hFOB1.19 cells treated with MSCs-derived exosomes were detected by cell count kit-8 (CCK-8) assay. The effect of MSCs-derived exosomes on cell apoptosis was evaluated by flow cytometry. Protein expression levels of apoptosis-related genes in hFOB1.19 cells were detected by Western blot.

RESULTS: MSCs differentiated into osteoblasts and lipoblasts under different treatments. Meanwhile, MSCs-derived exosomes exhibited typical elongated morphology after isolation and culture for 1 and 3 days, respectively. Functionally, MSCs-derived exosomes could promote the viability of hFOB1.19 cells, and significantly increase the expression level of GLUT3. In addition, MSCs-derived exosomes remarkably downregulated apoptosis-related genes and decreased apoptosis in hFOB1.19 cells.

CONCLUSIONS: MSCs-derived exosomes could promote osteoblast proliferation *via* inhibiting cell apoptosis, eventually improving osteoporosis.

Key Words

Bone marrow stem cells, Exosome, Apoptosis pathway, Osteoporosis, Osteoblasts, Proliferation.

Introduction

Osteoporosis is a condition in which the amount of bone tissue per unit volume decreases and bone becomes fragile. This may eventually lead to severe metabolic bone diseases. Marrow stem cells (MSCs) are considered as adult stem cells that have the potential to differentiate into osteoblasts, chondroblasts and lipoblasts[1]. Due to the advantages of convenient acquisition and low immunogenicity, MSCs are expected to become exciting regenerative seed cells nowadays[2]. However, there are still some limitations existing in MSCs treatment, including invasive collection, a small amount of isolated cells, age-dependence and strong *in vitro* expansion[3]. To find suitable alternatives to stem cells, in-depth studies on repairing damaged tissues and promoting tissue regeneration by stem cells have been carried out. Recent studies have proved that MSCs participate in the microenvironment around damaged tissues *via* exosomes. Meanwhile, MSCs-derived exosomes regulate cell proliferation, differentiation and apoptosis in damaged tissues, thereby indirectly repairing the lesions[4]. Functionally, MSCs-derived exosomes can stimulate immune response and induce bi-directional function of immune tolerance[5]. Therefore, we speculated whether MSCs derived exosomes could promote osteoblast proliferation *via* inhibiting cell apoptosis, eventually improving osteoporosis.

Materials and Methods

Reagents and Instruments

Glutamine, α-MEM, penicillin were obtained from Gibco (Grand Island, NY, USA); fetal bovine serum (FBS), 0.25% trypsin, type I collagenase, ascorbic acid, dexamethasone β-sodium glycerophosphate, GLUT3 antibody, methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Inverted phase contrast microscopy camera system was obtained from Nikon (Tokyo, Japan); scanning electron microscope was obtained from Hitachi (Tokyo, Japan); flow cytometry was purchased from Bio-Tek (Winooski, VT, USA).

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Cell Isolation and Culture

This study was approved by the Animal Ethics Committee of Liuzhou Worker's Hospital Animal Center. 3-week old Sprague-Dawley (SD) rats (Model Animal Research Center of Nanjing University, Nanjing, China) were first executed with cervical vertebra dislocation. Then the femur and tibia were collected under aseptic condition. Subsequently, the marrow cavity was washed with L-Dulbecco's Modified Eagle Medium (L-DMEM). After centrifugation at 1000 r/min for 5 min, MSCs were re-suspended in L-DMEM containing 10% FBS. MSCs were then seeded into 6-well plates at a density of 1×10^6/L. When the confluence was up to 80-90%, the cells were passaged with 0.25% trypsin.

Identification of MSCs Surface Antigen

Third-generation MSCs were digested with trypsin, followed by centrifugation at 1000 rpm for 3 min. Then the supernatant was discarded, and the cells were washed with phosphate-buffered saline (PBS) for 2-3 times. CD34 and CD90 antibodies were diluted with PBS and added to cells for 30 min-incubation. The cell suspension was then centrifuged at 1000 rpm for 3 min, and the supernatant was discarded. Subsequently, the suspension was transferred to a special detection tube, followed by cell surface antigen determination by flow cytometry.

Osteogenesis and Lipid Differentiation of MSCs

Third-generation MSCs were first seeded into 24-well plates at a density of 5×10^4/mL. Osteogenesis differentiation was induced by α-MEM containing 5×10^{-5} mol/L isobutyl xanthine, 2×10^{-4} mol/indomethacin, 1×10^{-5} mol/L dexamethasone and 10 mg/L insulin. Meanwhile, lipid differentiation was induced by α-MEM containing 1×10^{-2} mol/L β-glycerophosphate, 1×10^{-8} mol/L dexamethasone and 50 μg/mL Vitamin C. Alizarin red staining was performed 14 days after osteogenesis induction, while oil red staining was conducted 21 days after lipid differentiation.

Collection and Identification of MSCs-derived Exosomes

Tunable resistive pulse sensing (TRPS) was used to detect the size distribution of MSCs-derived exosomes. A three-dimensional map corresponding to the diameter, concentration and scattering intensity of exosomes was introduced. The video and image information of exosome motion was preserved to further analyze the size distribution of exosomes derived from MSCs.

Western Blot

Total protein of hFOB1.19 cells treated with MSCs-derived exosomes was extracted by radio-immunoprecipitation assay (RIPA) solution (Yeasen, Shanghai, China). The concentration of extracted protein was determined by the bicinchoninic acid (BCA) kit (Abcam, Cambridge, MA, USA). Briefly, total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk for 1 h, the membranes were incubated with specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After washing with Tris-Buffered Saline and Tween-20 (TBST; Yeasen, Shanghai, China) 3 times, the membranes were incubated with the corresponding secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method. Relative protein expression levels were reflected by target protein/reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (gray value).

Cell Counting Kit-8 (CCK-8) Assay

hFOB1.19 cells were collected and seeded into 96-well plates at a dose of 1×10^5/ml. After culturing for 6, 24, 36, 48 and 72 h, 10 μL CCK-8 solution (Dojindo, Kumamoto, Japan) was added into each well, respectively, followed by incubation at 37°C for 2 h in the dark. Absorbance values at the wavelength of 490 nm were detected by a microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was repeated 3 times.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 Software (IBM, Armonk, NY, USA) was used for all statistical analysis. Quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used to compare the differences between the two groups. Least significant difference (LSD) analysis was performed for comparing the differences among different groups. $p<0.05$ was considered statistically significant.

Results

Phenotype Identification of MSCs

MSCs were in adherence after culturing for 1 day, and exhibited a significantly elongated morphology from the 3rd day (Figure 1A). Subsequently, flow cytometry was performed to identify MSCs surface antigens. Results showed significantly downregulated negative-antigen CD34 and upregulated positive-antigen CD90 (Figure 1B), suggesting the successful isolation of MSCs.

Identification of Multidirectional Differentiation of MSCs and MSCs-Derived Exosomes

Alizarin red staining results demonstrated that after osteogenesis differentiation for 14 days, calcified nodules of MSCs were accumulated (Figure 2A). Meanwhile, the expression of osteogenesis-related genes was remarkably elevated, including RUNX2 and ALP (Figure 2B). After lipid differentiation for 14 days, oil red staining also found that lipid droplets were accumulated (Figure 2C). Upregulated expression levels of lipid differentiation-related genes were also observed, including ADIPOQ and FABP4 (Figure 2D). TRPS results suggested that the size of exosome was mainly in 40-100 nm, which was consistent with the exosome morphology (Figure 2E). Subsequently, we detected the specific surface antigens (CD9 and CD63) by flow cytometry. The identification of CD9 and CD63 further confirmed the characteristics of exosomes.

MSCs-Derived Exosomes Promoted Osteoblast Proliferation

To further explore the effect of MSCs-derived exosomes on osteoblasts, CCK-8 assay was performed to detect the viability of hFOB1.19 cells. Results demonstrated that the viability of hFOB1.19 cells treated with MSCs-derived exosomes was significantly elevated (Figure 3A). Both the mRNA (Figure 3B) and protein (Figure 3C) levels of GLUT3 in hFOB1.19 cells treated with MSCs-derived exosomes were remarkably decreased. Besides, flow cytometry results demonstrated a significant reduction trend in cell apoptosis after treatment of MSCs-derived exosomes (Figure 3D). The above data all elucidated that MSCs-derived exosomes promoted osteoblast proliferation.

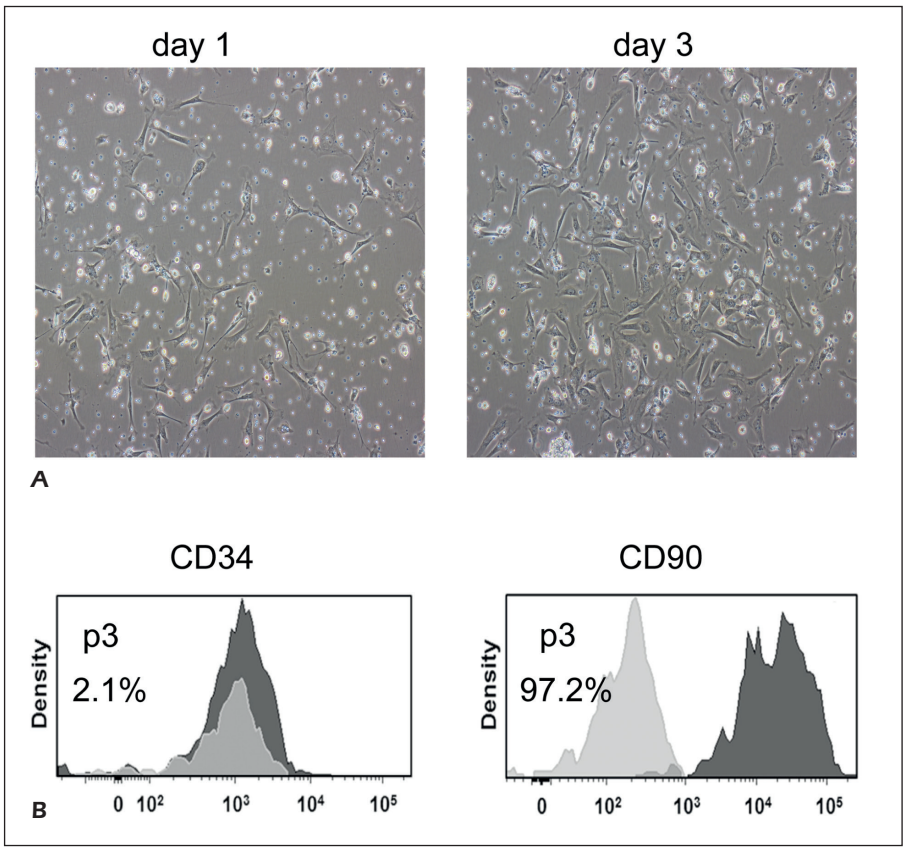

Figure 1. Phenotype identification of MSCs. **A**, Morphology of primary MSCs after culturing for 1 and 3 days, respectively. **B**, Negative-antigen CD34 and positive-antigen CD90 were identified by flow cytometry.

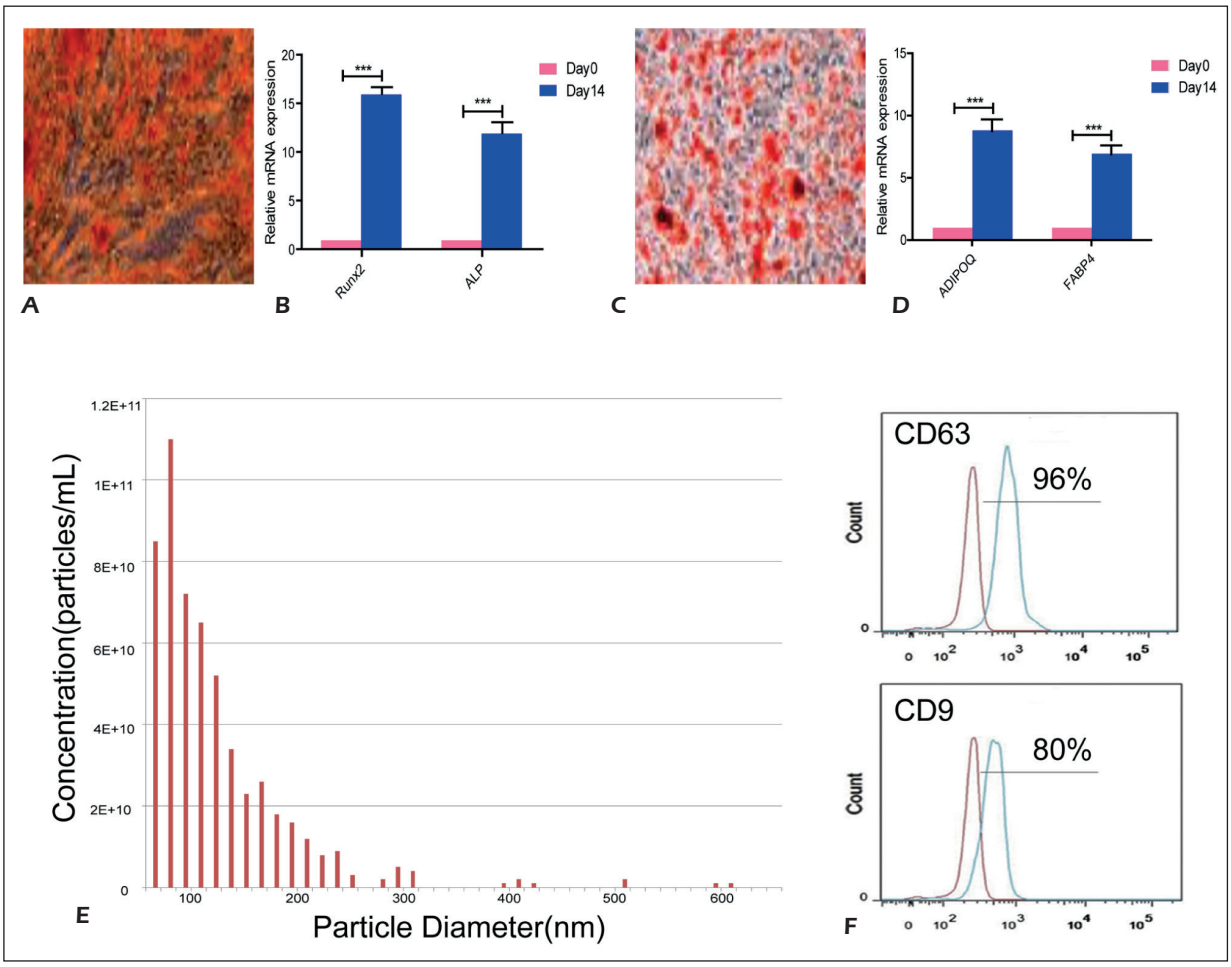

Figure 2. Identification of multidirectional differentiation of MSCs and MSCs-derived exosomes. A, Alizarin red staining results demonstrated osteogenesis differentiation. **B**, Osteogenesis-related genes (RUNX2 and ALP) were significantly upregulated after osteogenesis differentiation for 14 days. **C**, Oil red staining demonstrated lipid differentiation. **D**, Lipid differentiation-related genes (ADIPOQ and FABP4) were upregulated after lipid differentiation for 14 days. **E**, Size distribution of MSCs-derived exosomes were analyzed by TRPS. **F**, Specific surface antigens (CD9 and CD63) were identified by flow cytometry.

MSCs-Derived Exosomes Promoted Osteoblast Proliferation Via Inhibiting Cell Apoptosis

Subsequently, we explored the possible mechanism of MSCs-derived exosomes in promoting osteoblast proliferation. Western blot results demonstrated that the protein expression levels of Caspase-3 and Caspase-9 were significantly increased, whereas the expression levels of cleaved-Caspase-3 and cleaved-Caspase-9 were decreased (Figure 4A). This indicated that MSCs-derived exosomes could remarkably inhibit cell apoptosis. Furthermore, the elevated viability of hFOB1.19 cells treated with MSCs-derived exosomes could be rescued by si-cleaved-Caspase-3 transfection (Figure 4B), suggesting that MSCs-derived exosomes promoted osteoblast proliferation *via* inhibiting cell apoptosis.

Discussion

Osteoporosis is an orthopedic disease that seriously affects human health. It may also bring a long-term negative impact on patients and their families. MSCs are pluripotent stem cells with the potential to differentiate into multiple tissues. In recent years, MSCs have been widely applied in bone tissue reconstruction. In 2017, Zhu et al[6] isolated exosomes that were capable of repairing damaged tissues. Exosomes are a kind of extracellular vesicles that contain proteins, lipids and RNAs. Functionally, exosomes exchange cell-cell information by transferring biological substances to target cells[7]. Previous studies have reported that MSCs-derived exosomes can simulate most of the biological functions of MSCs, thereafter effectively promoting bone tissue engineering

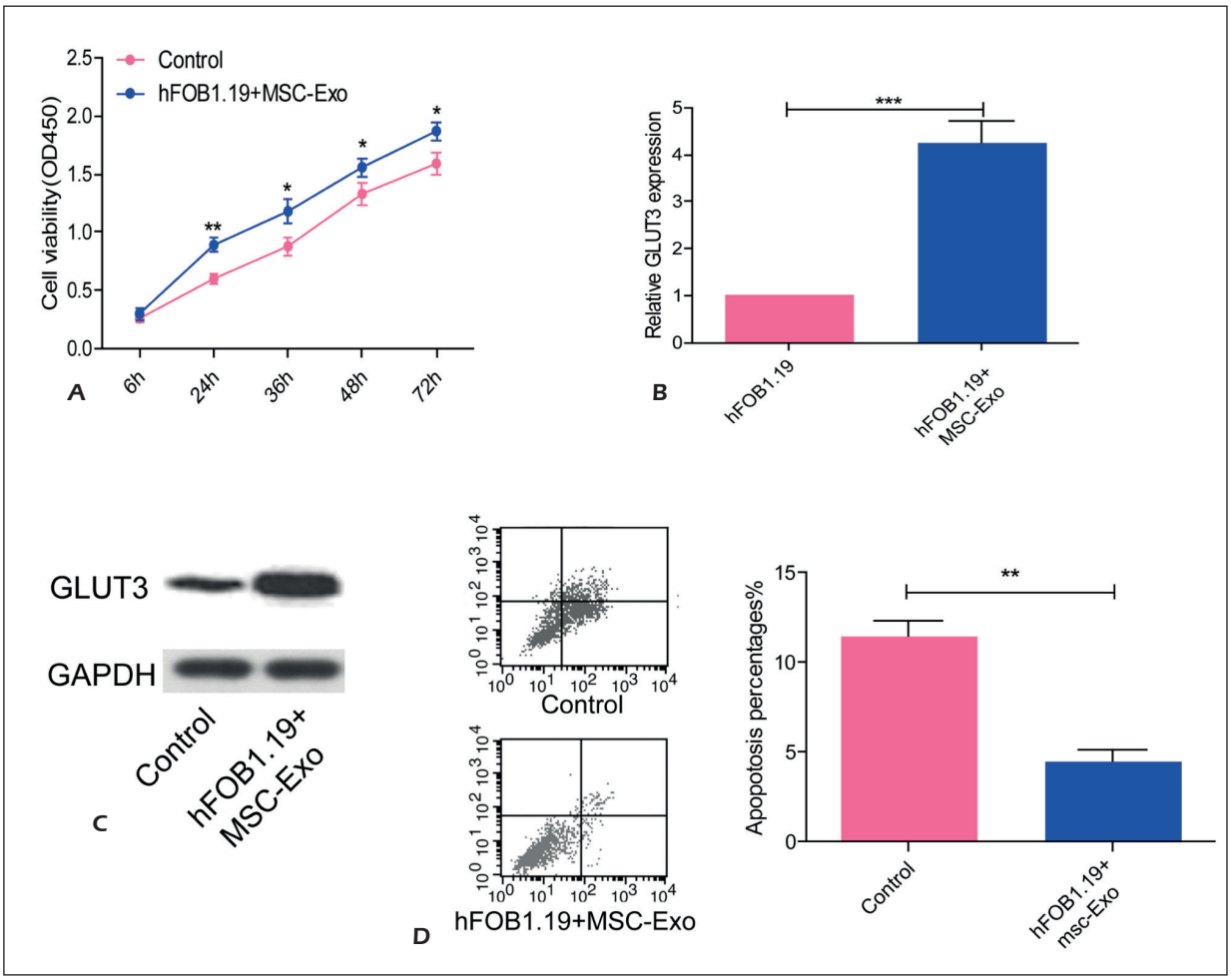

Figure 3. MSCs-derived exosomes promoted osteoblast proliferation. **A**, The viability of hFOB1.19 cells treated with MSCs-derived exosomes was remarkably increased. **B-C**, The mRNA (**B**) and protein (**C**) expression levels of GLUT3 in hFOB1.19 cells treated with MSCs-derived exosomes were significantly decreased. **D**, Cell apoptosis was remarkably decreased after treatment of MSCs-derived exosomes.

and injury repair. Therefore, MSCs-derived exosomes are expected to be widely applied in clinical practice.

MSCs-derived exosomes have been found to exert their function in promoting osteogenesis differentiation and bone tissue engineering repair through different ways[8,9]. For example, exosomes inhibit cell apoptosis in osteoporosis tissues *via* downregulating Bax[10], which is consistent with the results of Wu et al[11]. Meanwhile, MSCs-derived exosomes can regulate the immune system. Zhu et al[12] have proved that exosomes can regulate immune response by upregulating anti-inflammatory cytokines. In addition, Liu et al[13,14] have demonstrated that exosomes may enhance blood perfusion in lesion sites *via* promoting angiogenesis.

In the present study, we found that exosomes gradually entered the cytoplasm of MSCs to exert their biological roles in a time-dependent manner. It can be speculated that exosomes are served as an intercellular messenger. Exosomes contain osteogenesis-related proteins such as GLUT3, which is one of the important markers of osteoblast differentiation and maturation. In this study, CCK-8 results showed that exosomes could significantly promote the viability of hFOB1.19 cells. Meanwhile, the mRNA and protein expression levels of GLUT3 in hFOB1.19 cells were remarkably upregulated after treatment with MSCs-derived exosomes. Furthermore, MSCs-derived exosomes increased osteogenesis whereas decreased lipid differentiation, indicating that MSCs differentiated into osteoblasts.

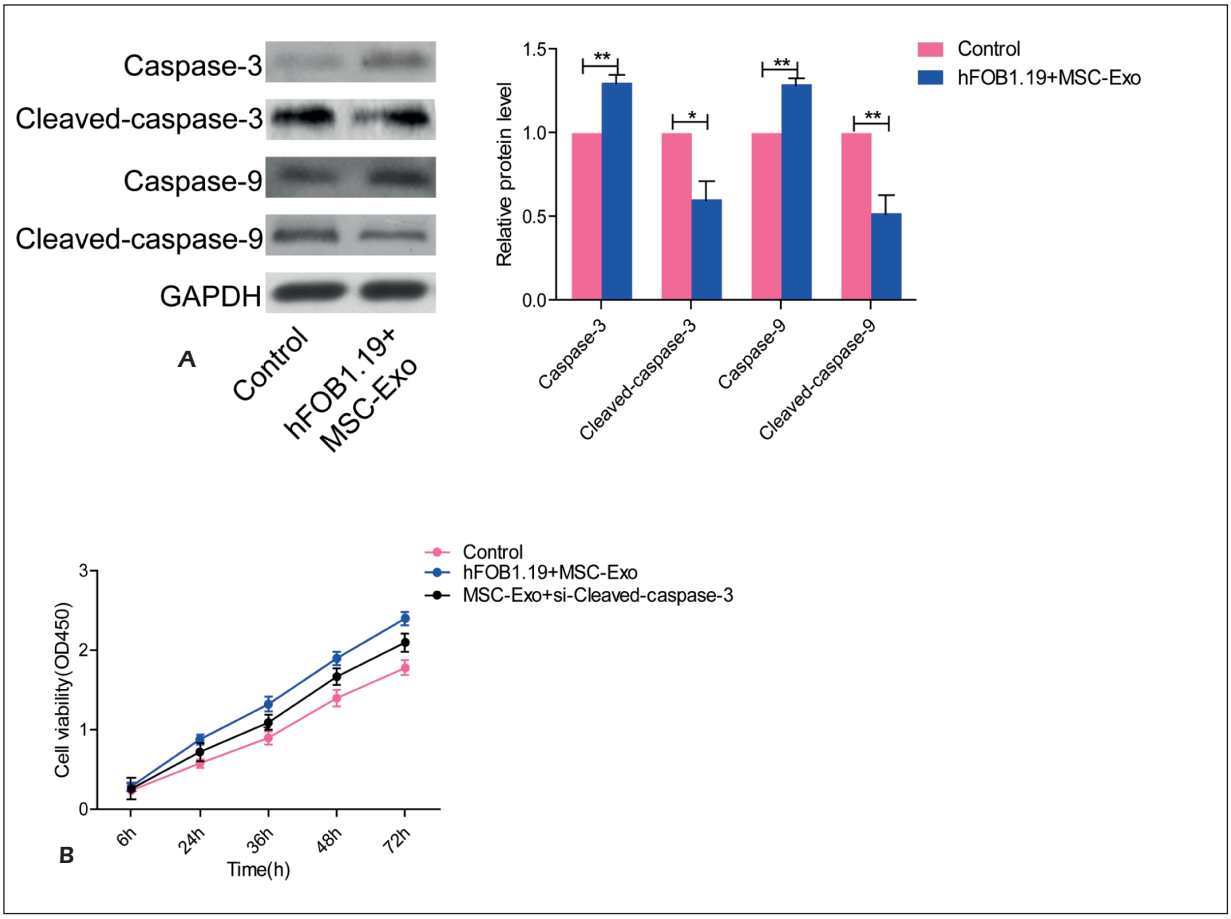

Figure 4. MSCs-derived exosomes promoted osteoblast proliferation via inhibiting cell apoptosis. **A**, Expression levels of apoptosis-related genes after MSCs-derived exosomes treatment were detected by Western blot. **B**, Elevated viability of hFOB1.19 cells treated with MSCs-derived exosomes could be rescued by si-cleaved-Caspase-3 transfection.

Scholars[1,15-17] have shown that exogenous MSCs participate in tissue regeneration through homing and differentiation. Only less than 1% of MSCs can be found in the lesion sites[18]. Consequently, researchers speculate that MSCs may promote tissue repair by secreting nutrient factors in endocrine and paracrine manners, which is further proved to be exosomes[19]. However, it remains unclear whether exosomes exert their repairing function by inhibiting cell apoptosis[8,20]. In the present study, *in vitro* experiments demonstrated that MSCs-derived exosomes could promote osteoblast proliferation by inhibiting the apoptosis signaling pathway.

To sum up, our study found that exosomes promoted the osteogenesis differentiation of MSCs. MSCs-derived exosomes inhibited cell apoptosis while promoted osteoblast proliferation. However, the osteoporosis process is complex. Various parameters of osteogenesis (such as ALP, BMP2, Wnt/β catenin, type I collagen, osteocalcin) play roles in the process of osteoporosis. In-depth studies are still needed to further explore some of those mechanisms in the future. This may eventually help to provide a theoretical basis for clinical treatment of osteoporosis.

Conclusions

We found that MSCs-derived exosomes could promote osteoblast proliferation *via* inhibiting cell apoptosis, eventually improving osteoporosis.

Conflict of Interests

The authors declare that they have no conflict of interest.

Acknowledgements

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