Abstract. – OBJECTIVE: Osteoporosis is the most common bone metabolic disease. Exosome exerts a crucial role in the development of multiple diseases. The aim of the study was to investigate the role of exosome derived from bone marrow mesenchymal stem cells (MSCs) in osteoporosis and its underlying mechanism.

MATERIALS AND METHODS: MSCs were first isolated from rat bone marrow. After the surface antigen of MSCs was identified by flow cytometry, MSCs-derived exosome (MSC-Exo) was extracted. The osteogenic and lipid differentiation abilities of BMSCs were determined by alizarin red staining and oil red staining, respectively. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to detect the mRNA expressions of genes. Cell counting kit-8 (CCK-8) assay was used to detect the viability of hFOB 1.19 cells. Western blot was used to measure expressions of the specific surface markers in exosomes and the MAPK pathway-related proteins in hFOB 1.19 cells. Moreover, cell cycle of hFOB 1.19 was detected by flow cytometry.

RESULTS: We observed a positive identification of surface antigens in MSCs, which presented good multidirectional differentiation ability. The isolated MSC-Exo exhibited typical morphology and particle size of exosomes, and the detection of specific surface labeled protein was positive under an electron microscope. After co-culture of MSC-Exo and osteoblast cell line hFOB 1.19, we found that MSC-Exo could promote the proliferation of hFOB 1.19 cells. Moreover, cell cycle of hFOB 1.19 was detected by flow cytometry.

CONCLUSIONS: MSC-derived exosomes improve osteoporosis by promoting the proliferation of osteoblasts via MAPK pathway.

Key Words
Bone marrow mesenchymal stem cells, Exosome, MAPK, Osteoporosis.

Introduction
Osteoporosis is a systemic bone disease characterized by weakened strength of bone structure, reduced bone mass, destroyed bone microstructures, increased bone brittleness and fracture risk. The incidence of osteoporosis increases with age1,2. With the growing aging of the world population, osteoporosis has become one of the most common and expensive diseases worldwide3. Osteoporosis is generally divided into two major categories, namely primary osteoporosis and secondary osteoporosis. The former type is further classified into postmenopausal osteoporosis, which is the most common type of primary osteoporosis, and senile osteoporosis. Osteoporosis patients are prone to experience fracture, and it may even cause death in serious cases. Moreover, osteoporosis seriously affects the health and life quality of these patients, resulting in a heavy burden and a series of major social problems. Therefore, it is urgent to seek for safe and effective prevention and control of osteoporosis.

Exosome, as an important part of the microenvironment, is a membrane vesicle secreted by most cells with 40-150 nm in diameter. After fusion of the polycystic body of various living cells and cell membrane, exosome is released...
Exosome derived from bone marrow mesenchymal stem cells improves osteoporosis

Exosome was firstly discovered in 1983, which was previously considered to be only a waste of cells. However, researchers have found that there are complex components of a variety of cells in this tiny membrane vesicle. For example, some proteins and lipid components with specific functions are distributed on the surface of the vesicle. Proteins in exosomes can be divided into two categories. The first is broad-spectrum proteins, which are correlated with the biological origin and function of exosomes, such as antigen binding proteins. The second is specific proteins, which present specific cellular functions. Physiological functions of exosomes with different sources are varied. Exosomes transport important biomolecules, such as receptors and ligands, to the target cells, which in turn trigger the release of these biomolecules.

Exosome exerts a crucial role in multiple diseases. Ono et al. have found that miR-23b is upregulated after BM2 cells are treated with MSC-Exo, thereby inducing dormancy through inhibiting the target gene MARCKS. Inhibited MARCKS eventually promoted the dormancy of breast cancer cells. Huang et al. have indicated that MSC-Exo is involved in the heart and blood vessels, which exerts effects of anti-apoptosis, cardiac regeneration, anti-cardiac remodeling, anti-inflammatory, new blood vessels formation and anti-vascular remodeling. Moreover, it is considered as a new potential molecular mechanism of MSCs transplantation therapy. In summary, there is a close relationship between exosomes and diseases. The research of exosome in osteoporosis, however, is relatively limited. Therefore, it is of great significance and value to explore the role of bone marrow derived exosome in osteoporosis.

Materials and Methods

Cell Extraction and Culture (MSCs and hFOB 1.19)

This study was approved by the Animal Ethics Committee of Jiangsu University Animal Center. 3-weeks old Sprague Dawley (SD) rats (Model Animal Research Center of Nanjing University, Nanjing, China) were executed with dislocation of cervical vertebra. The femur and tibia were collected under aseptic condition. The marrow cavity was washed with high glucose Modified Eagle Medium (MEM) medium (Gibco, Rockville, MD, USA) for collecting bone marrow cells (BMCs). BMCs were cultured in the Dulbecco’s Modified Eagle Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS), 1% L-glutamic acid and 1% double antibiotics (HyClone, South Logan, UT, USA). The BMCs were maintained in a 5% CO2 incubator at 37°C. The culture medium was firstly replaced 4 h later, followed by the replacement every two days. BMCs were digested with trypsin (Beyotime, Shanghai, China) for passage when the cell confluence was up to 80%. Second-passage BMCs were inoculated into the 6-well plates (3×10^5/mL) and the 24-well plates (7×10^4/mL), respectively.

Meanwhile, the hFOB 1.19 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% FBS, and were maintained in a 5% CO2 incubator at 37°C. The culture medium was replaced according to cell growth. Cell passage was performed when the cell confluence was up to 80% and the cells were reseeded into the 6-well plates (2×10^5/mL). Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Identification of MSCs Surface Antigen

Third-passage MSCs were digested with trypsin, followed by centrifugation at 1000 rpm for 3 min. The supernatant was discarded, and the cells were washed with phosphate-buffered saline (PBS, Yeasen, Shanghai, China) for 2-3 times. Then, the CD34 and CD90 antibodies were diluted with PBS and added to the cells for 30 min-incubation. Cell suspension was then centrifuged at 1000 rpm for 3 min, and the supernatant was discarded. Subsequently, cell suspension was transferred into a special detection tube, followed by the detection of cell surface antigen using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Osteogenesis and Lipid Differentiation of MSCs

The MSCs were seeded into the 6-well and 24-well plates, respectively. Osteogenesis differentiation was induced by high glucose MEM medium containing 10% fetal bovine serum (FBS), 1% L-glutamic acid, 1% double antibiotics, 0.25 mM ascorbic acid, 10 mM β-phosphoglycerol and 10 mM dexamethasone. Lipid differentiation was induced by high glucose MEM medium con-
taining 10% FBS, 1% L-glutamamide, 1% double antibiotics, 10 mM 3-isobutyl-1-methylxanthine, 10 mM indomethacin and 10 nM dexamethasone (HyClone, South Logan, UT, USA).

**Alizarin Red Staining**

The MSCs were seeded into the 24-well plates. After osteogenesis induction for 14 days, 60% isopropanol (GenePharma, Shanghai, China) was added to fix cells for 60 s, followed by phosphate-buffered saline (PBS) wash for 2 min. Subsequently, 10% alizarin red dyestuff (Yeasen, Shanghai, China) was used for staining for 10 min, followed by PBS wash for 3 times. The fluid was discarded, and an optical microscope (Olympus, Tokyo, Japan) was used for observation and image collection.

**Oil Red Staining**

The MSCs were seeded into the 24-well plates. After lipid induction for 14 days, 4% formalin solution was added to fix cells for 15 min, and 0.5% oil red O (Yeasen, Shanghai, China) was added for 40 min incubation. The working fluid was discarded, and the plates were washed with 60% isopropanol. An optical microscope was used for observation and image collection.

**Exosome Extraction**

The culture medium of MSCs was collected, and cell supernatants were centrifuged at 300 g for 10 min and 2000 g for 15 min for removing residual cells. Cell debris were further removed by centrifugation at 12000 g for 30 min. Filtration was then performed using 0.22 µm to remove particles larger than 200 nm. The supernatant was centrifuged at 100000 g for 2 h. Subsequently, cell suspensions were centrifuged at 100000 g for 2 h again, and the supernatants were discarded. Finally, 100 µL of PBS buffer were used for re-suspension, followed by the preservation at -80°C. All the above steps were carried out at 4°C.

**Cell Counting Kit-8 (CCK-8) Assay**

The hFOB 1.19 cells were collected and seeded into the 96-well plates at a dose of 6×10^3/mL. Briefly, 10 µL of CCK-8 solution (Dojindo, Kumamoto, Japan) were added into each well after cell culture for 6 h, 24 h, 36 h, 48 h and 72 h, respectively. Cells were incubated at 37°C for 2 h in the dark. Absorbance values at the wavelength of 490 nm were detected by the microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was repeated for 3 times.

**Cell Cycle Assay**

The hFOB 1.19 cells American Type Culture Collection (ATCC, Manassas, VA, USA) were seeded into the 6-well plates. After cell culture for 24 h, the cells were collected and washed with PBS for 3 times. Cells were immobilized with precooled 70% ethanol, and Rnase was added and incubated for 15 min. After that, 50 µg/mL of propidium iodide (PI) (Bio-Rad, Hercules, CA, USA) were added to each sample and incubated for 30 min. The cell cycle was detected by flow cytometry. Each experiment was repeated for 3 times.

**Real-Time Fluorescence Quantitative Polymerase Chain Reaction (qRT-PCR)**

We used TRIzol kit (Yeasen, Shanghai, China) to extract total RNA of the hFOB 1.19 cells. The extracted mRNA was reversely transcribed to complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Primers used in this study were showed in Table I.

**Western Blot**

The total protein of hFOB 1.19 cells treated with exosomes was extracted by the radioimmunoprecipitation assay (RIPA) lysate (Yeasen,}

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>GLUT3</td>
<td>5'-CGGCTTCTACCCATCACTCTTC-3'</td>
<td>5'-GGCACGACTTAGACATGG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AGGAGGCGAGATCCCGCAACA-3'</td>
<td>5'-GGCCGTCAGCGCCACATCTT-3'</td>
</tr>
<tr>
<td>ALP</td>
<td>5'-GGGACCTTGGTACTCGGACACAAT-3'</td>
<td>5'-GGCCCTCTCATCAGTTGATCT-3'</td>
</tr>
<tr>
<td>Bglap</td>
<td>5'-CATGAGGAGCCCTCTCTCCTTG-3'</td>
<td>5'-TGACATGAAGCCCTTGTTGCA-3'</td>
</tr>
<tr>
<td>Runx2</td>
<td>5'-GCACCCAGGCGGATATAAAC-3'</td>
<td>5'-TTGGACAGCAAGAACCACCC-3'</td>
</tr>
<tr>
<td>PPARG</td>
<td>5'-TTATTGACCCAGAAGGAC-3'</td>
<td>5'-ACCGACAGGGTCCACAGAG-3'</td>
</tr>
<tr>
<td>CEBPA</td>
<td>5'-GATCTACTCCCGTCTGATCACTG-3'</td>
<td>5'-GATCCAGGGTACCTTTGGCCACACACACATC-3'</td>
</tr>
<tr>
<td>KLF5</td>
<td>ACACCGAGCGCCAGCTTCA-3'</td>
<td>5'-TCCATTGGCTGCTGTCTGATTCTAG-3'</td>
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Table I. qRT-PCR primer pairs.
Shanghai, China). The concentration of each protein sample was determined by a bicinchoninic acid (BCA) kit (Abcam, Cambridge, MA, USA). Briefly, total protein was separated by a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel under denaturing conditions and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight. After washing with Tris-buffered saline and tween 20 (TBST, Yeasen, Shanghai, China) for 3 times, membranes were incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method, and the relative protein expression levels were reflected by target protein/reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (gray value).

**Statistical Analysis**

We used Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) for statistical analysis. GraphPadPrism5.0 (GraphPad Software Inc., La Jolla, CA, USA) was applied for image editing. The t-test was used for comparison between two groups. *p*<0.05 was considered statistically significant (*p*<0.05, **p**<0.01, and ***p**<0.001).

**Results**

**Phenotype Identification**

After MSCs inoculation for 1 and 3 days, some of adhered cells exhibited a vortex distribution. Cell morphology was diverse, which was manifested as sharp boundary and good refraction (Figure 1A). Expressions of cell surface antigens CD34 and CD90 were detected by flow cytometry. Results showed negative-antigen CD34 (1.5%) and positive-antigen CD90 (99.8%), indicating the high purity of the extracted MSCs (Figure 1B).

![Figure 1. Morphology and identification of MSCs. A. The primary BMSCs (after 1 and 3-days growth) showed a long spindle shape. B. BMSC’s surface antigen identification by flow cytometry. Negative antigen: CD34; positive antigen: CD90.](image)
Identification of Multidirectional Differentiation of MSCs and MSC-Exo

Second-passage cells were inoculated into the 24-well plates for induction of osteogenesis and lipid differentiation, respectively. After induction for 14 days, alizarin red staining results indicated that there were a large number of calcified nodules (Figure 2A). The total mRNA of cells at different time points of osteogenesis induction was extracted for detecting expression levels of ALP, BGLAP and RUNX2. We found that the expressions of these genes were remarkably increased in a time-dependent manner (Figure 2B). Similarly, oil red staining results also showed that there were a large number of lipid droplets (Figure 2A). Meanwhile, total mRNA of cells underwent lipid induction with different time points was extracted for detecting expression levels of PPARG, CEBPA and KLF5, which were remarkably increased in a time-dependent manner (Figure 2D).

The extracted exosome was observed under an electron microscope, and the diameter was about 40 nm (Figure 2E). Relative proteins were extracted from exosomes, and results demonstrated that specific proteins of CD9, CD63 and CD81 were positively expressed (Figure 2F), indicating that the extracted exosome was in accordance with the standard and could be used for subsequent experiments.

MSC-Exo Promoted the Proliferation of hFOB 1.19 Cells

After cells were treated with MSC-Exo, we detected cell viability of hFOB 1.19 at 6 h, 24 h, 36 h, 48 h and 72 h, respectively. Our findings suggested that the cell viability was remarkably elevated.

Figure 2. Identification of multidirectional differentiation of MSCs and MSC-Exo. A, Osteogenesis differentiation ability was detected by alizarin red staining. B, The expression changes of osteogenic related genes after culture in the osteogenesis induction medium for different days. C, Lipid differentiation ability was detected by oil red staining. D, The expression changes of lipid related genes after culture in the lipid induction medium for different days. E, Representative images of the MSCs-Exo under an electron microscope. F, The expressions of specific surface markers of exosomes (CD9, CD63 and CD81) were detected by Western blot.
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after MSC-Exo treatment (Figure 3A). Moreover, mRNA and protein expressions of GLUT3 in cells treated with MSC-Exo group were remarkably higher than that of the controls (Figure 3B-C). Subsequently, we used flow cytometry to detect cell cycle and found that MSC-Exo treatment remarkably promoted cell cycle when compared with that of negative controls (Figure 3D). These results all suggested that MSC-Exo promotes the proliferation of hFOB 1.19 cells.

**MSC-Exo Promoted the Proliferation of hFOB 1.19 Cells Through MAPK Pathway**

The cells were divided into the blank control group and the MSC-Exo group. After cell culture for 24 h, expression levels of key proteins in MAPK pathway were detected. Experimental results indicated that p-p38 and p-JNK were significantly upregulated in the MSC-Exo group (Figure 4A), suggesting that MSC-Exo activated the MAPK pathway. The cells were further divided into three groups, including the blank control group, single MSC-Exo group and MSC-Exo+JNK knockdown group. After cell culture for 24 h, CCK-8 assay demonstrated that cell viability of single MSC-Exo group was higher than that of the blank control group and MSC-Exo+JNK knockdown group (Figure 4B). Subsequently, we further detected cell cycle of the three groups. Results showed that cell cycle of the single MSC-Exo group was promoted when compared with that of blank control group. However, cell cycle of the MSC-Exo+JNK knockdown group was remarkably blocked than that of the single MSC-Exo group (Figure 4C). The above results suggested that MSC-Exo promotes the proliferation of hFOB 1.19 cells via MAPK pathway.
Discussion

MSCs are stem cells that remain in adult tissues, which have the ability of infinite amplification and multi-differentiation. Due to the advantages of convenient obtain, low immunogenicity, no ethical controversy and secretion of nutritional factors, MSCs are expected to be an exciting seed cell line in regenerative medicine\(^{16}\). Various researchers have believed that MSCs not only replace the damaged tissues through self-proliferation and differentiation, but also change the microenvironment of damaged tissues through the regulation of an important micro-environmental regulator by paracrine. Functionally, MSCs regulate cell proliferation, differentiation, apoptosis and other biological functions, and indirectly exert a therapeutic effect on repairing\(^{17}\). Nearly all cells can secrete exosomes, and the components of exosomes with different cell sources are varied. Therefore, exosomes are considered as important mediators of local microenvironment, and a vital component of stem cells for the repair of many organs as well\(^{18}\). Nowadays, multiple researches have reported that exosomes derived from MSCs have similar functions as MSCs, including repairing damaged tissues, inhibiting inflammatory reaction and regulating immune responses. Compared with MSCs, MSCs-Exo exhibits many advantages, such as good stability, easy preservation, no immunogenicity, easy access and convenient transformation. Thus, it

![Figure 4. MSCs-Exo promoted the proliferation of osteoblasts through MAPK pathway. A, The expressions of related proteins in hFOB 1.19 cells were detected by Western blot. B, The viability changes of hFOB 1.19 cells were detected by CCK-8 assay. C, The cell cycle of hFOB 1.19 cells was detected by flow cytometry.](image-url)
provides a new way for the treatment of many diseases\(^9\). We suggested that MSCs-Exo with the above characteristics may be an ideal substitute for improving the deficiency of traditional MSCs therapy in the repair of osteoporosis.

Mitogen-activated protein kinase (MAPK) signaling occurs in many cells, which participates in cell survival, proliferation and differentiation\(^20\). Previous investigations\(^21\) have shown that gene expression and function of osteoblasts are related to the stimulation of MAPK signaling. Thouverey et al\(^22\) have found that intermittent parathyroid hormone (iPTH) can increase bone mass and intensity by stimulating the number and activity of osteoblasts. Meanwhile, PTH exerts an anabolic effect in mature osteoblast and bone cells via the cAMP/protein kinase A (PKA) signaling pathway.

In addition, it has been found that bone mineral density showed a significant and gradual decrease in osteoblast specific p38 knockout mice at the age of 5 weeks. The volume of bone trabeculae in these mice decreased by 62% at the age of 6 months. In mutant mice, there was also a gradual decrease in the cortical thickness of long bones. These abnormalities are associated with a decrease of endothelial cells, trabecular formation rate, and the expressions of type 1 collagen, alkaline phosphatase, osteopontin and osteocalcin\(^23\).

Based on the characteristics of exosomes and the important role of the MAPK signaling pathway in osteoblasts, we conducted a series of researches on exploring the mechanism of MSC-Exo and MAPK pathway in osteoblasts. In this study, we first isolated the original MSCs for exosome extraction. After treated with MSC-Exo, the proliferation and cell cycle of hFOB 1.19 were significantly promoted. At the same time, we noticed that MSC-Exo could increase the expressions of related proteins in MAPK pathway. Therefore, we suspected that exosome might regulate the proliferation of hFOB 1.19 cells through MAPK pathway. The cells were then divided into three groups, including the blank control group, single MSC-Exo group and MSC-Exo+JNK knockdown group. Results demonstrated that cell viability of single MSC-Exo group was higher than that of the blank control group, and cell cycle of the single MSC-Exo group was promoted when compared with the blank control group. Meanwhile, cell viability of the MSC-Exo+JNK knockdown group was significantly blocked. From the above results, we may conclude that MSC-Exo can promote the proliferation of hFOB 1.19 cells through MAPK pathway, thus relieving osteoporosis. However, the relationship between MSC-Exo and osteoporosis remains to be further analyzed.

## Conclusions

We showed that MSC-Exo could promote the proliferation of hFOB 1.19 through MAPK signaling pathway, thus alleviating the progression of osteoporosis.

## Conflict of interest

The authors declared no conflict of interest.

## References


