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Bone marrow stem cells-derived exosomes extracted from osteoporosis patients inhibit osteogenesis via microRNA-21/SMAD7

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Abstract. – OBJECTIVE: To explore whether bone marrow stem cells (MSCs)-derived exosomes extracted from osteoporosis patients could inhibit osteogenesis via microRNA-21/SMAD7.

PATIENTS AND METHODS: MSCs from osteoporosis patients were isolated and cultured. MSCs morphology was observed, and the specific surface antigens were identified by flow cytometry. The osteogenic ability of MSCs was detected by alizarin red staining and oil red staining. Exosomes were collected from MSCs suspension by ultracentrifugation, and microRNA-21 expression in MSCs derived-exosomes was detected. Moreover, protein and mRNA levels of ALP, Bglap, and Runx2 in MSCs treated with different sources of MSCs-derived exosomes were detected by qRT-PCR (quantitative real-time polymerase chain reaction) and Western blot, respectively. ALP activity in MSCs was accessed by a relative commercial kit. Furthermore, binding sites of microRNA-21 and SMAD7 were predicted by Targetscan, miRWalk, and miRDB, and were further verified by luciferase reporter gene assay. SMAD7 expression in MSCs derived-exosomes was also detected.

RESULTS: MSCs extracted from healthy adults, and osteoporosis patients were in adherent growth and exhibited elongated morphology, which could differentiate into osteoblasts and lipoblasts after different inductions. MicroRNA-21 expression in MSCs-derived exosomes extracted from osteoporosis patients was remarkably higher than those extracted from healthy adults. Decreased Runx2 expression and ALP activity were found after treatment of MSCs-derived exosomes extracted from osteoporosis patients. SMAD7 was confirmed to bind to microRNA-21 and was downregulated in osteoporosis patients in comparison with healthy adults. Overexpression of SMAD7 resulted in downregulated ALP, Bglap, and Runx2.

CONCLUSIONS: MicroRNA-21 inhibits osteogenesis through regulating MSCs-derived exosomes extracted from osteoporosis patients via targeting SMAD7.

Key Words:
Osteoporosis, Bone marrow mesenchymal stem cells, Exosomes, MicroRNA-21, SMAD7, Osteogenesis.

Introduction

Osteoporosis is a kind of bone metabolic disease with high mortality and morbidity. Osteoporosis seriously threatens the physical health of the elderly, especially in postmenopausal women[1]. Current osteoporosis treatment is unable to fundamentally reverse the occurrence of osteoporosis. MSCs are the seed cells of bone regeneration, which exert a crucial role in maintaining normal bone metabolism. Dysfunction of osteogenic differentiation is an important reason for the occurrence of osteoporosis[2,3]. Scholars have found that transplanted exogenous MSCs can reverse osteoporosis by regulating osteogenic differentiation of MSCs from host bone marrow. However, the specific regulatory mechanism of MSCs-derived exosomes is still not clear[4,5].

Some studies have found that transplantation of exogenous cells affects biological functions of host cells by secreting exosomes. Exosomes are a type of secretory bodies with the membrane structure that can stably transport microRNAs and other key signals[6,7]. In recent years, relative studies have pointed out that MSCs can replace damaged tissues through proliferation and differentiation. Besides, MSCs could indirectly repair damaged tissues through secreting exosomes in the microenvironment. MSCs derived-exosomes have a bidirectional function of activating the immune response and inducing the immune toleran-

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ce. Large amounts of proteins, lipids, and nucleic acid components contained in exosomes are capable of affecting multiple signaling pathways in target cells[8]. It is reported that microRNA-21 is overexpressed in estrogen-deficient patients with osteoporosis and estrogen-deficient mice. The underlying mechanism of microRNA-21 in osteoporosis development, however, has not been fully elucidated[9].

SMAD7 is a significant transcription factor in downstream pathways of bone morphogenetic proteins, which positively regulates osteogenic differentiation of osteoblasts induced by bone morphogenetic proteins. Relative reports have demonstrated that microRNA-21 can promote the proliferation of lung cancer cells A549 via targeting SMAD7. In our study, we explored the potential effect of SMAD7 on osteogenic differentiation of MSCs derived-exosomes extracted from osteoporosis patients, so as to provide a theoretical basis for better treatment of osteoporosis.

Patients and Methods

Reagents and Instruments

Glutamine, α-MEM, and penicillin were obtained from Gibco (Grand Island, NY, USA); fetal bovine serum (FBS) and microRNA-21 primers were obtained from Ribobio (Guangzhou, China). Antibodies of CD9, CD63, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 0.25% trypsin, type I collagenase, ascorbic acid, dexamethasone β-sodium glycerophosphate, GLUT3 antibody, and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA); quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) reagents were obtained from Applied Biosystem (Foster City, CA, 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 obtained from Bio-Tek (Winooski, Vermont, USA).

Cell Isolation and Culture

2 mL of bone marrow was extracted from osteoporosis patient and diluted in DMEM (Dulbecco's Modified Eagle Medium). After centrifugation at 500 r/min for 10 min, the supernatant and the upper layer of fat were discarded. We then added Ficoll lymphocyte separation solution, followed by centrifugation at 2,000 r/min for 30 min. BMSCs were collected and seeded in the 6-well plates at a density at 1×10^6/L. Culture medium was half-replaced within the first 24 h, and then completely replaced every two days. MSCs morphology was observed using an inverted microscope. Cell passage was performed with 0.25% trypsin when the cell confluence was up to 80-90%. Our investigation was approved by the Ethics Committee of Yantai Yeda Hospital. All the subjects signed the informed consent.

Identification of MSCs Surface Antigens

Third-passage MSCs were washed and re-suspended with phosphate-buffered saline (PBS). 2×10^5/mL MSCs were preserved in an EP tube, followed by incubation with 2 μL of CD34 and CD90 (1:500) at 4°C for 1 h. Subsequently, MSCs was washed and re-suspended in 200 μL of PBS for surface antigen identification using flow cytometry.

Identification of Osteogenesis and Lipid Differentiation

MSCs extracted from healthy adults and osteoporosis patients were seeded in 12-well plates at a density of 5×10^4/mL, respectively. Osteogenic differentiation was performed by adding α-MEM containing 5×10^{-5} mol/L isobutyl xanthine, 2×10^{-4} mol/L indomethacin, 1×10^{-5} mol/L dexamethasone and 10 mg/L insulin. Lipid differentiation was performed by adding α-MEM containing 1×10^{-2} mol/L β-glycerophosphate, 1×10^{-8} mol/L dexamethasone and 50 μg/mL Vitamin C. Differentiation induction solution was replaced every 3 days. Cells were fixed with 4% polyoxymethylene, stained with oil red solution for 15 min at room temperature and captured using a microscope after 21 days of lipid differentiation. Besides, cells were fixed with 4% polyoxymethylene, stained with 0.1% Tris-Hcl (pH=8.3) and captured using a microscope after 14 days of osteogenic differentiation.

Collection and Identification of MSCs-Derived Exosomes

MSCs extracted from healthy adults, and osteoporosis patients were seeded in a culture bottle at a density of 5×10^6/75 cm^2, and cultured in 10 ml of serum-free DMEM for 3 days, respectively. After centrifugation at 10,000 rpm/min for 30 min, the suspension was collected and 500 μL of exosome extraction solution was added for incubation at 4°C overnight. Furthermore, exosomes were precipitated after centrifugation at 10,000 rpm/min

for 1 h. Size distribution of MSCs-derived exosomes was detected using Tunable Resistive Pulse Sensing (TRPS).

Western Blot

The total protein of MSCs-derived exosomes was extracted by the radioimmunoprecipitation assay (RIPA) lysate (Yeasen, Shanghai, China). The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). Briefly, total protein was separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) under denaturing conditions and then transferred to PVDF (polyvinylidene difluoride) membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1h, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight. After washing 3 times with Tris-Buffered Saline and Tween 20 (TBST; Yeasen, Shanghai, China), membranes were incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method, and the relative protein expression levels were reflected by target protein/reference GAPDH (gray value).

ORT-PCR

The total RNA was extracted from cells by TRIzol method (Invitrogen, Carlsbad, CA, USA) and then transcribed into complementary Deoxyribose Nucleic Acid (cDNA). The reverse transcription reaction was carried out in strict accordance with the instructions of SYBR Green Real-Time PCR Master Mix (Invitrogen, Carlsbad, CA, USA), with a total reaction volume of 10 μL. The parameters of the thermal cycling were as follows: denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 5 min, for a total of 40 cycles. Each sample was repeated in triplicate.

Luciferase Reporter Gene Assay

The target genes of microRNA-21 were predicted by Targetscan, miRWalk, and miRDB, followed by verification using luciferase reporter gene assay. Briefly, MSCs in logarithmic growth period were seeded into 6-well plates at a density of 5×10^4/mL. MSCs were treated with MSCs-derived exosomes extracted from healthy adults and osteoporosis patients when the cell confluence was up to 60-70%, respectively according to the instructions of Lipofectamine 2000. Luciferase activity was determined using single photon detector (Bio-Rad, Hercules, CA, USA). Relative luciferase activity = firefly luciferase activity value / Renilla luciferase activity value.

ALP Activity Determination

MSCs were lysed and centrifuged at 12,000×g for 10 min, followed by ALP activity determination using ALP activity kit (Beyotime, Shanghai, China). Blank control well, standard well, and sample well were established, respectively. Based on the manufacturer's instructions, 4, 8, 16, 24, 32, and 40 μL of standard solution was added in the standard wells, respectively, whereas 50 μL of standard solution was added in the sample wells. After incubation at 37°C for 10 min, termination solution was added, followed by ALP activity determination at the wavelength of 405 nm.

Statistical Analysis

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

Results

Phenotype and Differentiation Identification of MSCs Extracted from Healthy Adults and Osteoporosis Patients

MSCs extracted from healthy adults exhibited elongated morphology after isolation and culture (Figure 1A, left). Alizarin red staining and oil red staining showed accumulated calcified nodules (Figure 1A, middle) and lipid droplets (Figure 1A, right), respectively. Similarly, MSCs extracted from osteoporosis patients showed typical morphology (Figure 1B, left), calcified nodules (Figure 1B, middle) and lipid droplets (Figure 1B, right) as well. The above data suggested the successful isolation of MSCs.

Identification of MSCs-Derived Exosomes

The diameter of exosomes derived from healthy adults (Figure 2A, left) and osteoporo-

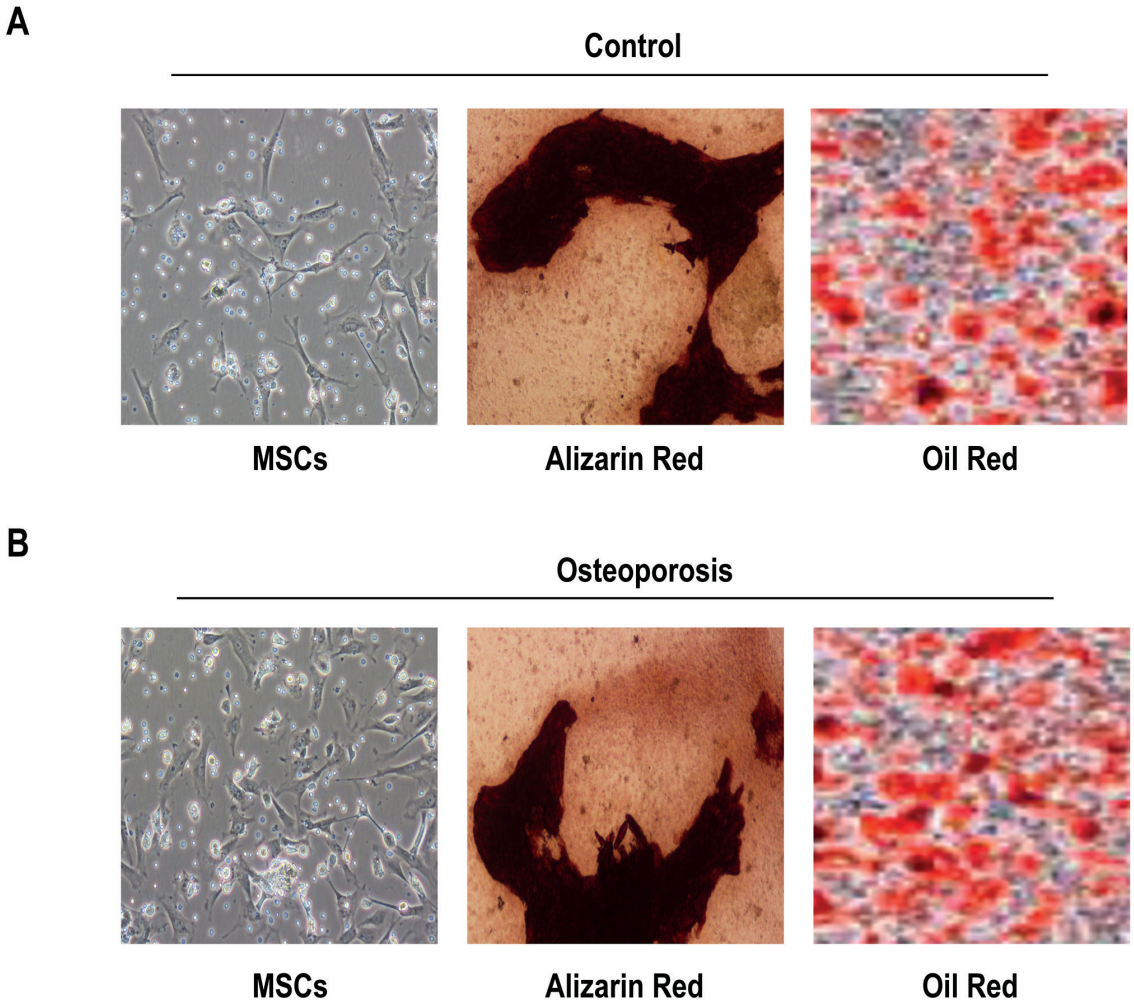

Figure 1. Phenotype and differentiation identification of MSCs extracted from healthy adults and osteoporosis patients. ***A***, Typical morphology (left), calcified nodules (middle) and lipid droplets (right) in MSCs extracted from healthy adults. ***B***, Typical morphology (left), calcified nodules (middle) and lipid droplets (right) in MSCs extracted from osteoporosis patients.

sis patients (Figure 2A, right) was 50-100 nm. MSCs-specific proteins were detected, and the results showed that CD9 and CD63 were overexpressed in exosomes, whereas barely expressed in cells (Figure 2B). QRT-PCR results elucidated that microRNA-21 was overexpressed in MSCs-derived exosomes extracted from osteoporosis patients than that of healthy adults (Figure 2C).

MSCs-Derived Exosomes Extracted from Osteoporosis Patients Inhibited MSCs Osteogenesis

MSCs were treated with exosomes derived from different populations for 7-day osteogenesis. QRT-PCR results elucidated that mRNA levels of ALP, Bglap, and Runx2 were remarkably lower in MSCs treated with exosomes derived from osteoporosis patients than those derived from healthy adults (Figure 3A-C). Decreased ALP activity was also observed in MSCs treated with exosomes derived from osteoporosis patients (Figure 3D). Moreover, protein expression of Runx2 in MSCs treated with exosomes derived from osteoporosis patients was remarkably lower than those derived from healthy adults (Figure 3E), indicating that MSCs-derived exosomes extracted from osteoporosis patients inhibited MSCs osteogenesis.

MicroRNA-21 Targeted to SMAD7

Bioinformatics predicted that microRNA-21 was bound to 3'-UTR of SMAD7 (Figure 4A). Further luciferase reporter gene assay verified that

microRNA-21 could bind to wild-type SMAD7 (Figure 4B). Subsequently, normal MSCs were treated with exosomes extracted from healthy adults and osteoporosis patients, respectively. QRT-PCR data demonstrated higher microRNA-21 but lower SMAD7 expression in MSCs treated with exosomes extracted from osteoporosis patients than those from healthy adults (Figure 4C and 4D), indicating that microRNA-21 directly targets to SMAD7.

MSCs-Derived Exosomes Extracted from Osteoporosis Patients Inhibited Osteogenesis Via MicroRNA-21/SMAD7

MSCs were divided into three groups according to different treatments, including MSCs treated with exosomes extracted from healthy adults, MSCs treated with exosomes extracted from osteoporosis patients, and MSCs treated with exosomes extracted from osteoporosis patients and SMAD7 overexpression. The mRNA levels of ALP, Bglap, and Runx2 were remarkably lower in MSCs treated with exosomes derived from osteoporosis patients than those derived from healthy adults. However, no significant differences in mRNA levels of these genes were found between MSCs treated with exosomes extracted from osteoporosis patients and SMAD7 overexpression, and those treated with healthy adults-derived exosomes (Figure 5A-C). ALP activity detection obtained the similar results (Figure 5D). Our results suggested that SMAD7 overexpression could partially reverse the inhibitory osteogenesis by MSCs-derived exosomes extracted from osteoporosis patients.

Discussion

According to the White Paper on Prevention and Treatment of Osteoporosis in China, there are at least 69.44 million people in China suf-

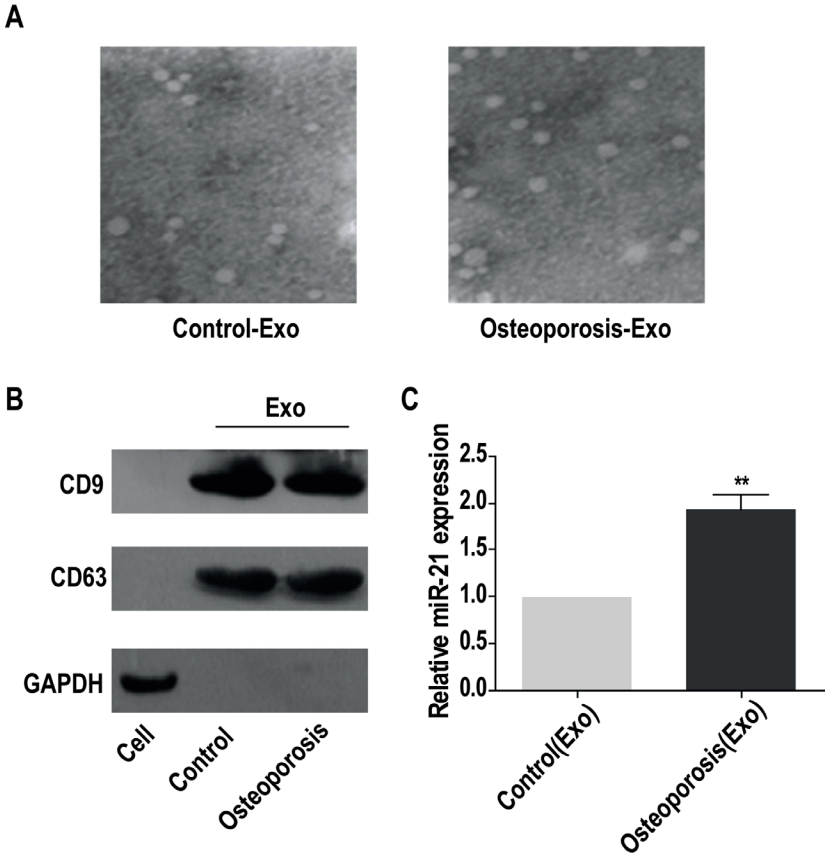

Figure 2. Identification of MSCs-derived exosomes. ***A,*** The diameter of exosomes derived from healthy adults (left) and osteoporosis patients (right) was 50-100 nm. ***B,*** CD9 and CD63 were overexpressed in exosomes, whereas barely expressed in cells. ***C,*** MicroRNA-21 was overexpressed in MSCs-derived exosomes extracted from osteoporosis patients than that of healthy adults.

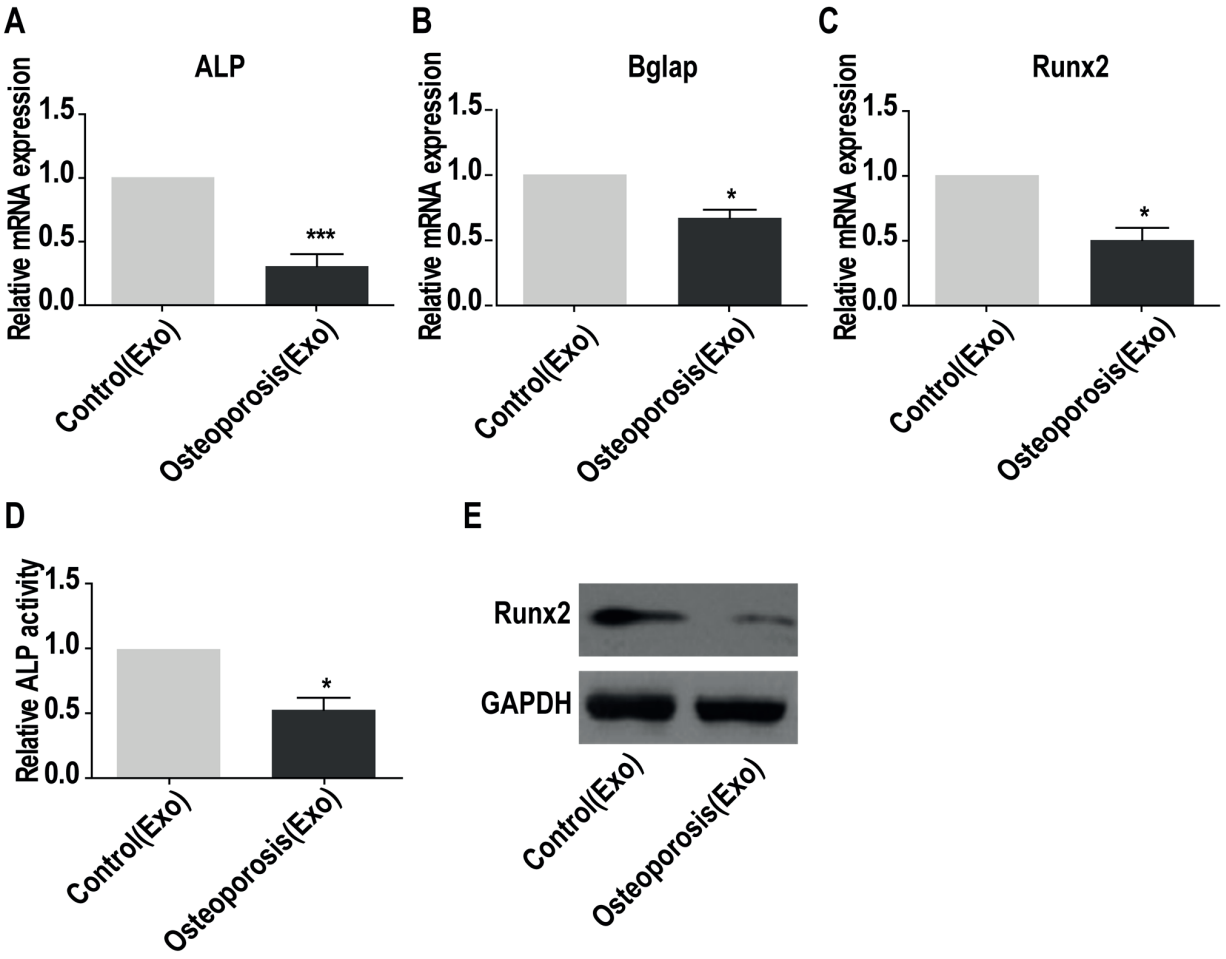

Figure 3. MSCs-derived exosomes extracted from osteoporosis patients inhibited MSCs osteogenesis. ***A-C,*** The mRNA levels of ALP (***A***), Bglap (***B***), and Runx2 (***C***) were remarkably lower in MSCs treated with exosomes derived from osteoporosis patients than those derived from healthy adults. ***D,*** Decreased ALP activity was observed in MSCs treated with exosomes derived from osteoporosis patients. E, Protein expression of Runx2 in MSCs treated with exosomes derived from osteoporosis patients was remarkably lower than those derived from healthy adults.

fering from osteoporosis. It is expected that the case number of patients with osteoporosis and low bone mass will increase to 280 million in 2020. However, there still lacks of safe and effective treatment. Current treatment can only inhibit bone resorption, but cannot reverse osteoporosis by promoting osteogenic recovery of bone mass[10]. Recent studies have shown that MSCs injection is capable of reversing osteoporosis through restoring host osteogenic function, which is expected to become an important approach for treating osteoporosis in the future. Transplantation of MSCs has been proved to be an effective treatment of many diseases. The specific mechanism, however, remains unclear[11]. Early studies have shown that transplanted MSCs can be directly differentiated into multiple types of cells to promote tissue regeneration. Further studies have found that few MSCs after transplantation are survival, whereas paracrine plays a key role in the therapeutic effect of MSCs. Recently, relative studies have shown that exosomes also exert a crucial role in MSCs treatment[12,13].

It is reported that microRNA-21 participates in the differentiation and proliferation of stem cells, which also controls the adipogenic differentiation of MSCs. However, the mechanism by which microRNA-21 regulates the proliferation and differentiation of MSCs needs to be further explored[14]. SMAD7 is an essential control gene in osteoblast osteogenesis and differentiation,

which promotes the expression levels of osteoblast markers. SMAD7 deficiency leads to bone dysplasia or developmental termination[15,16]. In the present work, we found that 3'-UTR of SMAD7 was complementarily paired with microRNA-21, suggesting that SMAD7 is the downstream gene of microRNA-21. SMAD7 is an important transcription factor in the downstream pathway of bone morphogenetic proteins. Studies[17-19] have confirmed that SMAD7 positively regulates osteogenic differentiation. Therefore, we speculated whether microRNA-21 affects the osteogenic differentiation via regulating SMAD7, which was then verified by luciferase reporter gene assay.

Although it has been experimentally confirmed that SMAD7 is a downstream target of microRNA-21, the role of which in inhibiting osteogenesis of osteoporosis patients still needs to be verified[20]. Here, MSCs were divided into three groups according to different treatments, including MSCs treated with exosomes extracted from healthy adults, MSCs treated with exosomes extracted from osteoporosis patients and MSCs treated with exosomes extracted from osteoporosis patients and SMAD7 overexpression. Osteogenic capacity in each group was observed. It is concluded that SMAD7 overexpression inhibits the effect of exosomes derived from osteoporosis patients on osteogenesis. The data suggested that SMAD7 blocks the osteogenesis suppression by microRNA-21, which indirectly demonstrated that SMAD7 is an essential target of microRNA-21.

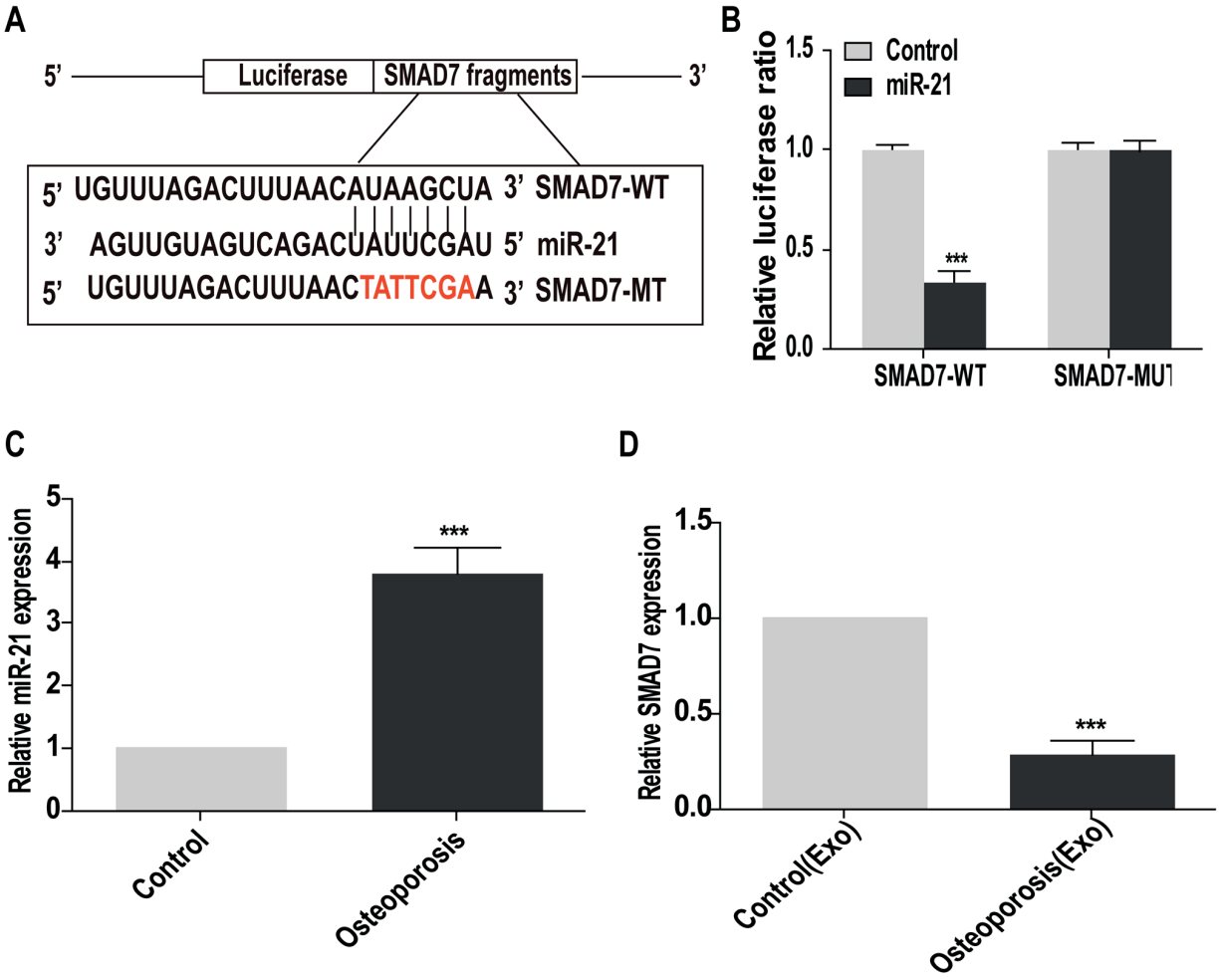

Figure 4. MicroRNA-21 targeted to SMAD7. ***A,*** Bioinformatics predicted that microRNA-21 was bound to 3'-UTR of SMAD7. ***B,*** Luciferase reporter gene assay verified that microRNA-21 could bind to wild-type SMAD7. ***C-D,*** Higher microRNA-21 (***C***) but lower SMAD7 expression (***D***) in MSCs treated with exosomes extracted from osteoporosis patients than those from healthy adults.

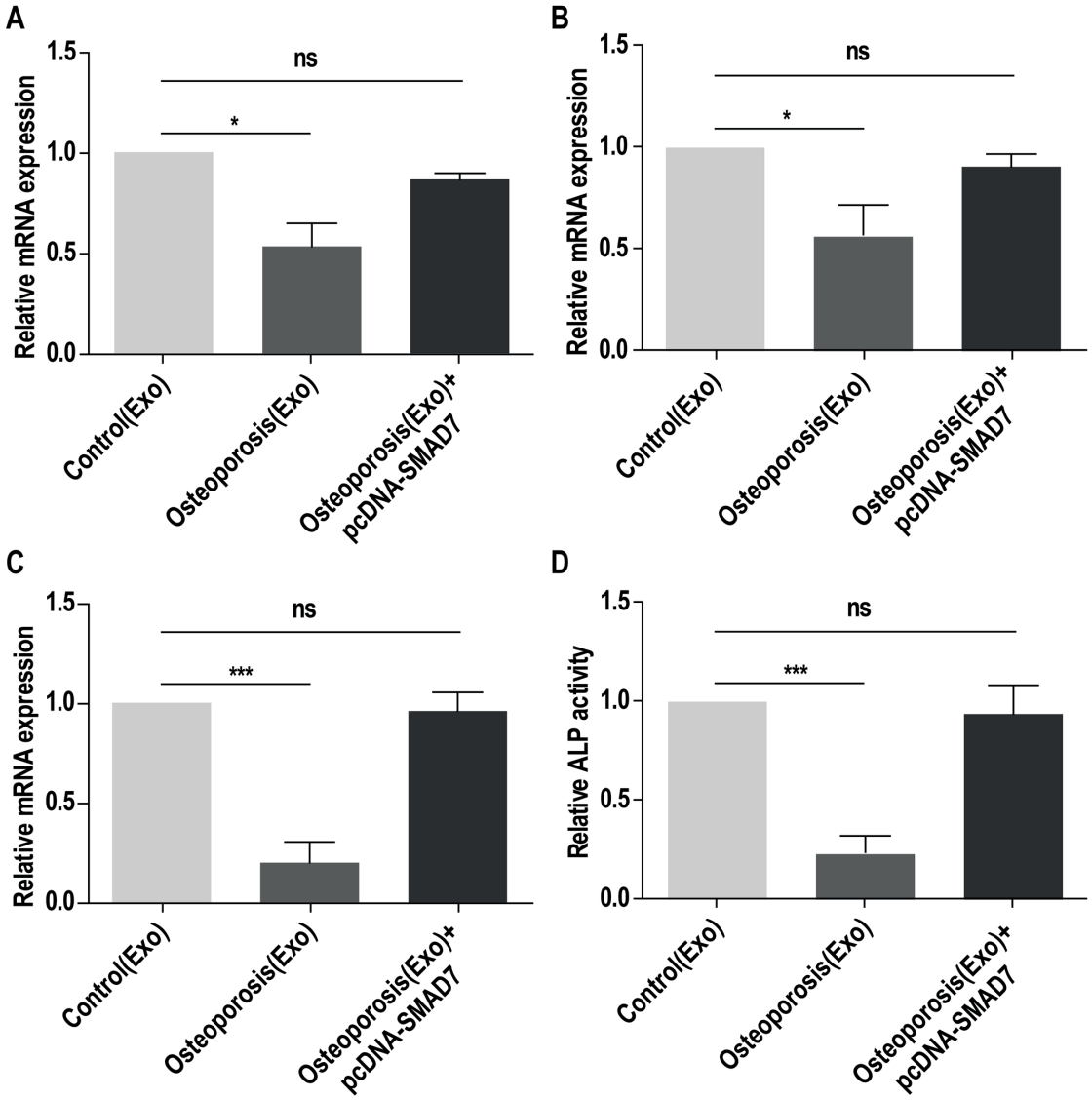

Figure 5. MSCs-derived exosomes extracted from osteoporosis patients inhibited osteogenesis via microRNA-21/SMAD7. ***A-C,*** The mRNA levels of ALP (***A***), Bglap (***B***) and Runx2 (***C***) were remarkably lower in MSCs treated with exosomes derived from osteoporosis patients than those derived from healthy adults. No significant difference in mRNA levels of these genes was found between MSCs treated with exosomes extracted from osteoporosis patients and SMAD7 overexpression, and those treated with healthy adults-derived exosomes. ***D,*** ALP activity detection was remarkably lower in MSCs treated with exosomes derived from osteoporosis patients than that derived from healthy adults.

To sum up, microRNA-21 is downregulated in MSCs-derived exosomes extracted from osteoporosis patients, which inhibits osteogenesis via binding to SMAD7. Our results provide a new direction in exosome application, so as to avoid risks of immune-related rejection resulted from stem cell transplantation, acquired gene mutations, instable gene expression, and tumor development.

Conclusions

We showed that microRNA-21 inhibited osteogenesis through regulating MSCs-derived exosomes extracted from osteoporosis patients via targeting SMAD7.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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