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RESEARCH ARTICLE

BMP-2 transgenic alveolar bone-derived marrow stem cells attenuate osteonecrosis of the femoral head

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Abstract

***Background*:** Osteonecrosis of the femoral head (ONFH) results in activity-related hip pain and disability, affecting around 20 million people worldwide. However, there is absent of effective therapeutic approaches for ONFH. The transplantation of mesenchymal stem cells was reported to be a promising method for ONFH therapy. In this study, we explored if bone morphogenetic protein-2 (BMP-2) transgenic alveolar bone-derived marrow stem cells (ABMSCs) could be applied to treat ONFH.

***Methods*:** We established an ONFH mouse model by injection of steroid, and implanted ABMSCs and BMP-2 transgenic ABMSCs into the steroid-impaired femoral head. Gene expression and the pathological alternation of the tissues were analyzed by qRT-PCR and hematoxylin and eosin staining, respectively. Tartrate-resistant acid phosphatase staining was applied to detect osteoclastogenesis. Enzyme linked immunosorbent assay (ELISA) and Western blot were performed to evaluate the protein expression in the sera and tissues, respectively.

***Results*:** ABMSCs attenuated steroid-induced ONFH and ameliorated the serum concentration of osteogenesis-associated proteins in the ONFH mice. Mechanistically, ABMSCs inhibited osteoclast differentiation by inactivating the RANKL/RANK/OPG signaling pathway. BMP-2 overexpression enhanced ABMSCs to alleviate ONFH.

***Conclusions*:** ABMSCs and BMP-2 Transgenic ABMSCs attenuate ONFH by promoting osteoblastogenesis and inhibiting osteoclastogenesis via inactivating the RANKL/RANK/OPG signaling pathway.

Keywords: *ABMSCs*; *BMP-2*; *ONFH*; *osteoclast*

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Osteonecrosis of the femoral head (ONFH) is a prevalent disease affecting the hip joint, which arises from the programmed cell death of endothelial cells and the death of bone marrow and osseous tissue in the femoral head (1, 2). The progressive pathology of ONFH leads to hip pain that is triggered by activity and can result in disability due to the collapse of the femoral head. There are around 20 million patients suffering from ONFH worldwide (3). However, effective therapeutic approaches for ONFH are absent. One of the reasons causing this situation is that the pathogenesis of osteonecrosis is not sufficiently elucidated.

The pathogenesis of ONFH remains elusive. There is a widely accepted belief that the cause of ONFH is the disruption of blood supply to the femoral head (4). Block of the blood flow to the femoral head stimulates cell death and disrupts the normal repair progress. The normal repair progress is triggered by angiogenesis in the necrotic bone, and then osteoclasts resorb the necrotic bone, followed by new bone generation by osteoblasts (5). In ONFH, femoral head collapse occurs following bone resorption by osteoclasts (4). The delicate regulation of the balance between the bone resorption activity of osteoclasts and the bone formation activity of osteoblasts is necessary for effective bone regeneration (6, 7).

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The differentiation and activation of osteoclasts are regulated by a signaling pathway that involves osteoprotegerin (OPG), the receptor activator of nuclear factor NF-κB (RANK), and RANK ligand (RANKL). RANK is activated by binding to RANKL and then elicits expression of osteoclastogenic genes, such as tartrate-resistant acid phosphatase (TRAP). The activated RANK makes osteoclasts polarization and secretion of the lytic enzymes TRAP and cathepsin K (CATK) to erode the necrotic bone. OPG behaves as an inhibitor and blocks the RANK/RANKL interaction to inhibit activation of the signaling pathway (8). Osteoblast differentiation is enhanced by cardiotrophin-1 (CT-1), insulin growth factor-1 (IGF-1), and bone morphogenetic protein-2 (BMP-2) (7). It is promising to manipulate osteoblastogenesis or osteoclatogenesis by intervention of these signaling pathways for ONFH therapy.

Bone marrow mesenchymal stem cells (BMSCs), being pluripotent stem cells, have the ability to differentiate into osteoblasts and stromal cells, which in turn provide support for hematopoiesis. BMSCs also regulate osteoclast activity to impact bone growth and remodeling (9). Considering the effective treatment of ONFH with the factors related to cell growth and differentiation, like BMPs and angiogenic growth factor (10), some investigators engineered BMSCs to overexpress BMP-2 and vascular endothelial growth factor (VEGF), and found these transgenic BMSCs are powerful to stimulate bone regeneration in the femoral head which had been destructed by ONFH (11, 12, 13). Alveolar bone-derived marrow stem cells (ABMSCs) are originated from tooth tissue and have a similar role as BMSCs to stimulate osteogenesis (14). Moreover, it is easier and safer to obtain ABMSCs than BMSCs.

Methods

Cell culture, lentivirus package and infection

The cells were cultured in a medium containing α-minimum essential medium (α-MEM), supplemented with 10% fetal bovine serum (FBS) from Welgene Inc., Republic of Korea, 10 mM L-ascorbic acid, and antibiotics from Gibco, Grand Island, NY. The cells were maintained in a Steri-Cycle 370 Incubator from Thermo Fisher Scientific, Waltham, MA, USA, which was kept humidified and at a temperature of 37°C with 5% CO_2. To facilitate overexpression of BMP-2 in ABMSCs, the full-length BMP-2 gene was amplified using PCR and inserted into the viral vector pLVX-IRES-ZsGreen1 from Clontech, CA, USA. Subsequently, this plasmid, along with plasmids PMD2.G and PSPAX2, was co-transfected into 293T cells to produce lentivirus particles containing the BMP-2 gene (Lenti-BMP-2). The ABMSCs were then infected with Lenti-BMP-2 particles at a multiplicity of infection (MOI) of 50 PFU/cell in the presence of 6 mg/ml polybrene from Sigma Aldrich, St. Louis, MO. Following infection for 24 h, the medium was replaced with fresh complete medium.

Osteogenic differentiation

Cells were seeded in 6-well plates at a density of 2×10^4 cells/cm^2. Two days later, the cells were induced to osteogenic differentiation in DMEM medium containing 5% FCS, 10 mM glycerophosphate, 50 μM L-ascorbic acid-2-phosphate and 10 nM DEX. The cells were further cultured for 10 days to complete differentiation and the medium was replaced with fresh differentiation medium every 2 days.

Quantitative reverse transcription polymerase chain reaction

The total RNA was purified using the PrimeScript RT Reagent Kit from TAKARA-BIO in accordance with the manufacturer's instructions and transcribed into complementary DNA (cDNA) using the Reverse Transcription System Kit from Invitrogen, Waltham, MA, USA. The expression levels were quantified using quantitative PCR with the SYBR Green Master Mix kit from ThermoFisher, Waltham, MA, USA, and the following primers: BSP, 5′-AAAGTGAGAACGGGGAACCT-3′ and 5′-GATGCAAAGCCAGAATGGAT-3′; OSC, 5′-GACTGTGACGAGTTGGCTGA-3′ and 5′-CTGGAGAGGAGCAGAACTGG-3′; Runx2, 5′-ACAACCACAGAACCACAAG-3′ and 5′-TCTCGGTGGCTGGTAGTGA-3′; β-actin, 5′-GCTCTCCAGAACATCACTCCTGCC-3′ and 5′-CGTTGTCATACCAGGAAATGAGCTT-3′. β-actin acted as an internal control.

Alizarin Red S Staining analysis of mineralization

To measure mineralization, the differentiated cells were stained with 2% Alizarin Red S (Sigma, St. Louis, MO) at pH 4.2 for 10 min, followed by washing with water. After mineral nodules were verified under a microscope, the dye in cells was extracted with 200 μl 10% acetic acid for 0.5 h. After a simple centrifuge, the recovered solution was heated at 85°C for 10 min, and then cooled down at 4°C. The solution was neutralized using 75 μl 10% ammonium hydroxide. The concentration of Alizarin Red S was determined by optical absorbance at 405 nm.

An ONFH mouse model and treatment of ONFH with ABMSC transplantation

C57BL/6 male mice, aged 8–12 weeks and weighting 20–25 g, were subcutaneously injected with either 21 mg/kg methylprednisolone (Pfizer, Inc.) (the Model group) or an equivalent dose of normal saline (the sham group). After 1 week, 2×10^4 ABMSCs or ABMSCs overexpressing BMP-1 in 1 ml PBS were transplanted into femoral head as previously described (15). Briefly, mice were anaesthetized with injection of pentobarbitone sodium

(Sigma, St. Louis, MO). A posterolateral incision was made to expose the femoral head. An epidural catheter was placed into the femoral head to inject the cell suspension in 5 min. After injection, absorbable collage sponge plugs were used to close the portal. Four weeks later, the mice were sacrificed to examine the treatment efficiency. The study was approved by Cangzhou Hospital of Integrated Traditional Chinese Medicine and Western Medicine.

Tartrate-resistant acid phosphatase staining analysis of osteoclasts

TRAP staining was executed to present osteoclasts with TRAP staining kit (Sigma, St. Louis, MO) following the manufacturer's instruction. The staining was observed under a microscope and the images were taken. The cells positively stained by TRAP and with multinuclei were numbered as osteoclast cells.

Enzyme linked immunosorbent assay and Western blot analysis of protein levels

To determine the concentration of BGP, BAP and TRAP in serum, serum was fractioned from 5 ml blood. The protein levels were measured by ELISA kit (Quidel, CA, USA) according to the manufacturer's protocol.

The tissues were dissected and homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.5, 0.1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 150 mM NaCl) for Western blot analysis. The protein samples were subjected to electrophoresis in SDS-PAGE gels, and the separated proteins were transferred onto PVDF membranes from Millipore, Bedford, USA. After blocking with 5% non-fat milk, the membranes were incubated overnight at 4°C with primary antibodies against RANK from Cell Signaling Technology, MA, USA, RANKL from Millipore, Billerica, MA, USA, OPG from Santa Cruz, Dallas, TX, USA, and GAPDH from Santa Cruz, Dallas, TX, USA. The membranes were then washed three times with washing buffer and blotted with HRP-coupled secondary antibodies from Promega, Madison, WI, USA. Protein bands were detected using the Enhanced Chemiluminescence Detection System from ThermoFisher, USA.

Statistical analysis

In this study, the data were expressed as the mean value ± SD (standard deviation). *P* values were calculated by one-way ANOVA analysis followed by a Tukey's post hoc test, and $P < 0.05$ was defined as statistical significance difference.

Results

BMP-2 overexpression facilitates ABMSC osteogenic differentiation in vitro

To explore the role of BMP-2 in osteogenic differentiation of ABMSCs, first we isolated BMSCs and ABMSCs and induced them to differentiate into osteoblasts. In response to osteogenic differentiation, BMSCs and ABMSCs expressed similar mRNA levels of osteoblastic marker genes, including bone sialoprotein (BSP) (Fig. 1A), osteocalcin (OSC) (Fig. 1B) and RUNX2 (Fig. 1C). Moreover, the mineralization levels in differentiated BMSCs and ABMSCs were nearly equal (Fig. 1D). These results demonstrate that the osteogenic capacity of ABMSCs is comparable to that of BMSCs. Next, we overexpressed BMP-2 in ABMSCs and then performed osteogenic differentiation. Compared to control cells, ABMSCs overexpressing BMP-2 expressed higher mRNA levels of the osteoblastic marker genes (Fig. 1A–1C) under osteogenic induction. Consistently, BMP-2 overexpression enhanced mineralization (Fig. 1D). These data suggest that BMP-2 overexpression facilitates ABMSC osteogenic differentiation *in vitro*.

AMBSCs and BMP-2 transgenic ABMSCs attenuates steroid-induced ONFH in mice

Since BMP-2 overexpression enhanced ABMSC differentiation into osteoblasts, it is interesting to examine the ONFH therapeutic effects of BMP-2 transgenic ABMSCs. An ONFH mouse model was established by injection with methylprednisolone, a steroid hormone. As shown in Fig. 2A, hematopoietic tissue nearly disappeared in the femoral head from the mice treated with steroid. Meanwhile, the ratio of empty lacunae was increased to over 40% in femoral head of the mouse treated with steroid (Fig. 2B). These are ONFH aspects, suggesting that the ONFH mouse model was successfully established. The hematopoietic tissue impaired by steroid treatment was partially recovered by ABMSCs transplantation (Fig. 2A, ABMSC). Compared with the ONFH model group, the ratio of empty lacunae was significantly decreased in the group of ABMSC transplantation (Fig. 2B). In line with the results that BMP-2 enhanced osteogenic differentiation, BMP-2 overexpression further recovered the impaired hematopoietic tissue (Fig. 2A, ABMSC + BMP-2) and decreased the ratio of empty lacunae (Fig. 2B). Interestingly, although steroid treatment did not affect the number of osteoblasts, both AMSCs and BMP-2 transgenic ABMSCs dramatically promoted the generation of osteoblasts (Fig. 2C). All together, these data indicate that ABMSCs attenuated steroid-induced ONFH and stimulated osteoblastogenesis, while these abilities of ABMSCs were strengthened by BMP-2 overexpression.

ABMSCs and BMP-2 transgenic ABMSCs inhibit osteoclast differentiation in mice with steroid-induced ONFH

As the importance of osteoclast activity in ONFH (16), we examined the effect of ABMSCs and BMP-2 transgenic ABMSCs on osteoclast activity. Osteoclasts can be stained in red by TRAP staining. As shown in Fig. 3A,

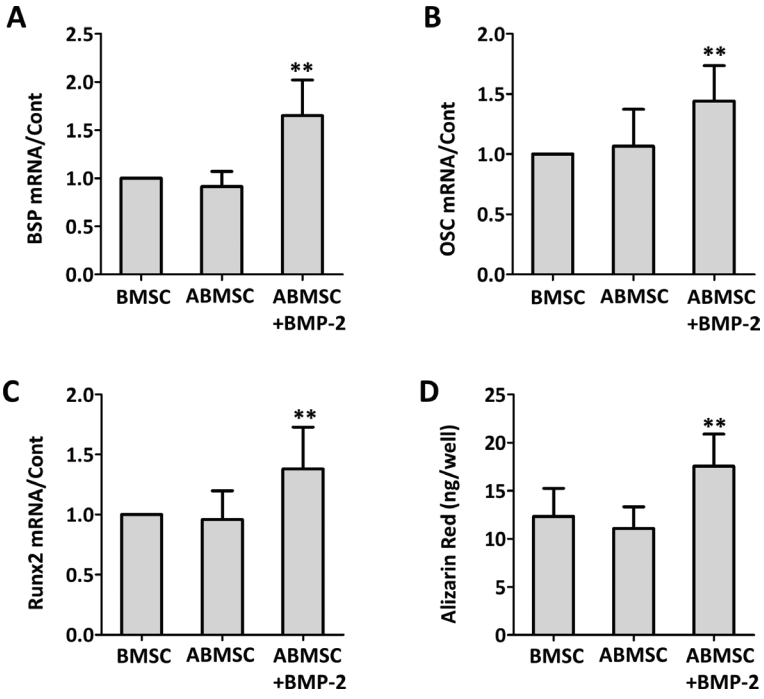

Fig. 1. BMP-2 overexpression facilitates ABMSC osteogenic differentiation *in vitro*. ABMSCs were isolated, and then infected with lentivirus particles containing the BMP-2 gene to establish BMP-2 transgenic ABMSCs. These cells were induced to osteogenic differentiation, followed by qRT-PCR analysis of mRNA levels of BSP (A), OSC (B) and Runx2 (C), and Alizarin Red S staining analysis of mineralization (D). ** $P < 0.01$, compared with BMSCs.

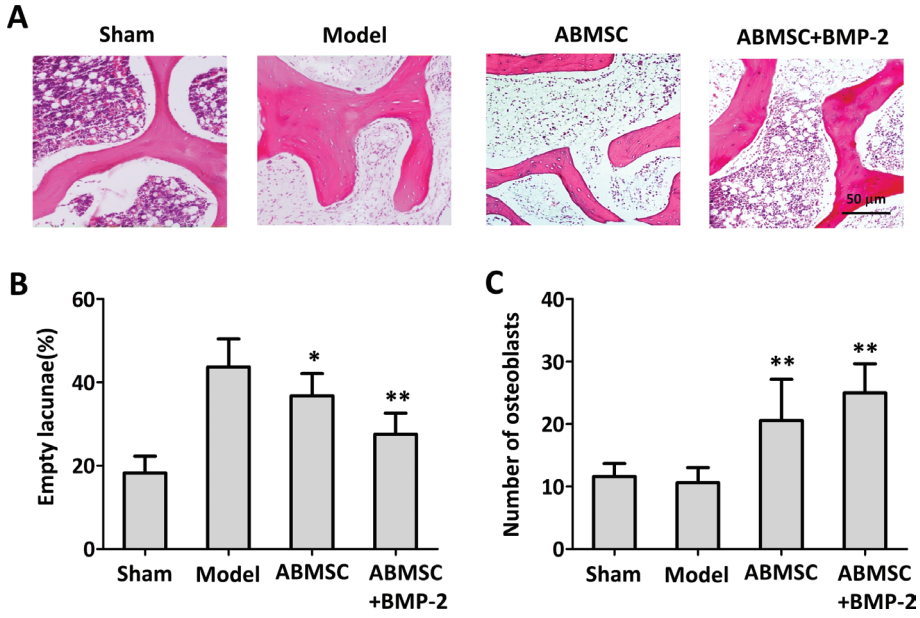

Fig. 2. ABMSCs and BMP-2 transgenic ABMSCs attenuate steroid-induced ONFH in mice. Mice were injected with methylprednisolone or normal saline, and 1 week later, ABMSCs were transplanted into the femoral head. After 4 weeks, the femurs were isolated, followed by H&E staining (A), and analysis the ratio of empty lacunae (B) and the number of osteoblasts (C) based on the results of H&E staining. *P <0.05, ** $P < 0.01$, compared with the ONFH model group.

steroid treatment dramatically increased the cells positively stained by TRAP, while the number of TRAP-positive cells was reduced by ABMSCs, and reduced more by BMP-2 transgenic ABMSCs. Based on the TRAP staining results, the number of osteoclasts was elevated by steroid treatment over five folds (Fig. 3B). Compared to

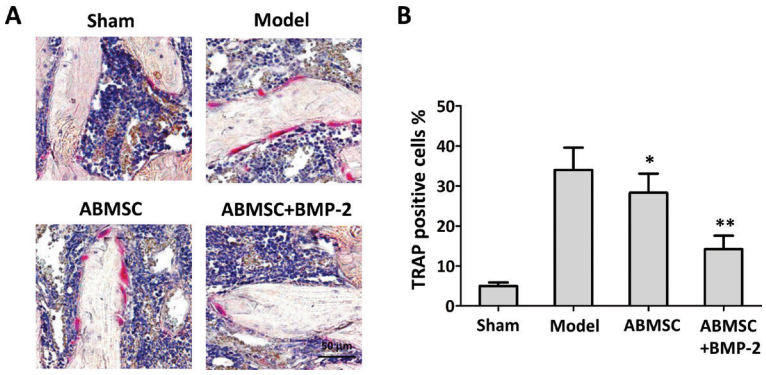

Fig. 3. ABMSCs and BMP-2 transgenic ABMSCs inhibit osteoclast differentiation in mice with steroid-induced ONFH. (A) Tartrate-resistant acid phosphatase (TRAP) staining to determine osteoclast activity in the femoral head of the indicated group of mice. (B) The number of osteoclasts which were positively stained and had multiple nuclei was calculated in the femoral head of the indicated mice. *$P < 0.05$, ** $P < 0.01$, compared with the ONFH model group.

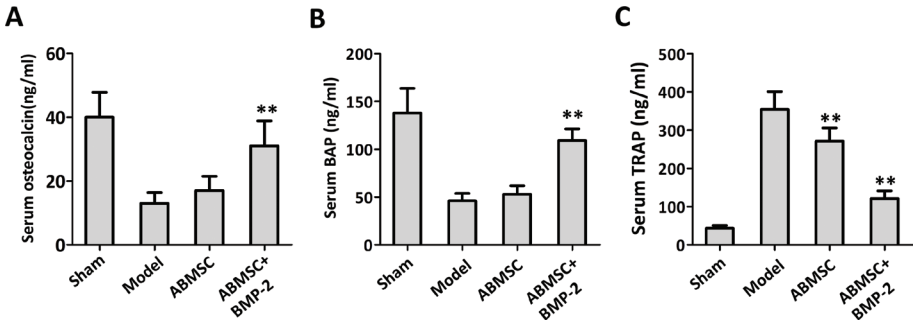

Fig. 4. ABMSCs and BMP-2 transgenic ABMSCs ameliorate the concentration of bone formation-associated proteins in mice with steroid-induced ONFH. ELISA analysis determined the concentration of BGP (A), BAP (B) and TRAP (C) in the sera of the indicated group of mice. *$P < 0.05$, ** $P < 0.01$, compared with the ONFH model group.

the ONFH model group, ABMSCs significantly decreased the number of osteoclasts, while BMP-2 transgenic ABMSCs reduced the number nearly three folds (Fig. 3B). These data imply that ABMSCs and BMP-2 transgenic ABMSCs inhibit osteoclast differentiation in mice with steroid-induced ONFH.

ABMSCs and BMP-2 transgenic ABMSCs ameliorate the concentration of bone formation-associated proteins in mice with steroid-induced ONFH

BGP and BAP are the serum markers of bone formation and TRAP is a serum marker of osteoclastogenesis (17). To confirm the conclusion that the transplantation of ABMSCs and BMP-2 transgenic ABMSCs attenuates ONFH, we examined the alternation of these serum markers in steroid-treated mice transplanted with ABMSCs or BMP-2 transgenic ABMSCs. Once ONFH was elicited by steroid, the serum levels of BAP and BGP were greatly declined (Fig. 4A and 4B), and in contrast, the serum level of TRAP was dramatically elevated (Fig. 4C). ABSMC transplantation did not have influence on the serum BAP concentration, but significantly increased the serum BGP amount and greatly reduced the serum TRAP level comparing with the ONFH model group (Fig. 4A-C). In agreement with the result that BMP-2 overexpression reinforced AMSC function on osteogenesis, BMP-2 strengthened ABMSC function to recover the serum BAP and BGP (Fig. 4A-B) and to reduce the serum TRAP (Fig. 4C). Based on these data, we confirm the conclusion that ONFH is alleviated by ABMSCs and BMP-2 transgenic ABMSCs.

ABMSCs and BMP-2 transgenic ABMSCs regulate the RANKL/RANK/OPG signaling pathway in mice with steroid-induced ONFH

Next, we explored the mechanism underlying which ABMSCs and BMP-2 transgenic ABMSCs inhibit osteoclast differentiation. The RANKL/RANK/OPG signaling pathway plays a critical role in osteoclastogenesis (8). Steroid treatment greatly induced expression of RANK and its ligand RANKL, which stimulates osteoclastogenesis, and dramatically inhibited expression of OPG, an

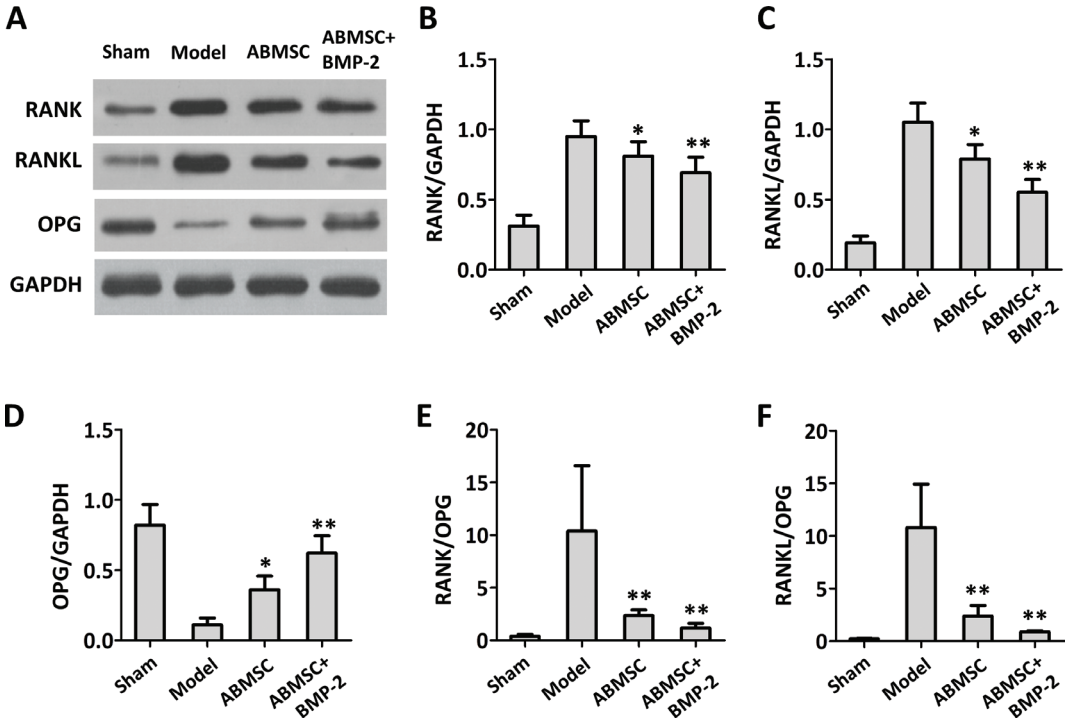

Fig. 5. ABMSCs and BMP-2 transgenic ABMSCs regulate the RANKL/RANK/OPG signaling pathway in mice with steroid-induced ONFH. (A) Western blot was carried out to detect the protein levels of RANKL, RANK, and OPG expression in the femoral heads of the indicated group of mice. The protein levels in (A) were quantified to calculate the ratio of RANK (B), RANKL (C) and OPG (D) to GAPDH, and the ratio of RANK/OPG (E) and RANKL/OPG (F). *$P < 0.05$, ** $P < 0.01$, compared with the ONFH model group.

antagonist of the RANKL/RANK signaling (Fig. 5A). ABMSCs slightly but significantly prevented steroid-induced expression of RANK and RANKL (Fig. 5A-C), and this influence was enhanced by BMP-2 overexpression (Fig. 5A-C). Steroid-impaired expression of OPG was partially restored by ABMSCs and nearly recovered by BMP-2 transgenic ABMSCs (Fig. 5A and 5D). The activity of the RANKL/RANK/OPG signaling is evaluated by the ratios of RANK/OPG and RANKL/OPG. As shown in Fig. 5E and 5F, steroid treatment tremendously increased the ratios of RANK/OPG and RANKL/OPG, which was partially blocked by ABMSCs and nearly completely prevented by BMP-2 transgenic ABMSCs. Collectively, these results demonstrate that ABMSCs and BMP-2 transgenic ABMSCs inhibit steroid activation of the RANKL/RANK/OPG signaling pathway.

Discussion

This study found that ABMSCs had comparable capacity as BMSCs to attenuate steroid-induced ONFH. Moreover, this capacity of ABMSCs was promoted by BMP-2 overexpression. Mechanistically, ABMSCs or BMP-2 transgenic ABMSCs promoted osteoblastogenesis and inhibited osteoclastogenesis by inactivating the RANKL/RANK/OPG signaling pathway.

There are several ONFH risk factors, and excessive steroid use is among them (18). Though the first case of steroid-induced ONFH was reported in 1953, the related pathologic process remains elusive (19). So far, multiple theories have arisen to explain steroid-induced ONFH (20). 1) Steroid alternates the lipid metabolism and leads to lipid deposition in bone cells to cause them death; 2) Steroid promotes BMSC adipogenesis and thereby declines the osteogenesis potential of BMSCs; 3) Steroid causes intravascular thrombosis to block blood supply in the bone, which induces bone cell death; 4) Steroid disturbs TLR4 signaling to elicit inflammation and activate osteoclasts. In this study, we found that the number of osteoclasts in the necrotic areas was significantly reduced by ABMSCs, suggesting the ABMSCs may inhibit steroid-elicited inflammation in ONFH. BMP-2 overexpression in ABMSCs further decreased the number of osteoclasts (Fig. 3B), consistent with the report that BMP-2 inhibits expression of IL-34, a pro-inflammatory cytokine (21). But there are contradictory reports showing that BMP-2 induces inflammation and the BMP-2-induced expression of the neuroinflammatory factors CGRP and SP benefits bone formation (22). These conflicting results may be associated with the doses of BMP-2, as it was reported that a high dose of recombinant

BMP-2 induces inflammation and does not benefit clinical outcomes of ONFH (23). In this study, BMP-2 was engineered into ABMSCs cells, and the expression level of BMP-2 can be regulated by ABMSCs. Although we did not examine the expression of the inflammatory genes, it assumes that BMP-2 is not expressed at a high inflammation-inducing level as BMP-2 enhanced ABMSCs amelioration of ONFH.

BMPs belong to the tumor growth factor-beta superfamily, possessing ability to inducing MSC differentiation into osteoblasts (24). BMP-2, one of 20-member-contained BMP subgroup, has been widely used for healing bone lesions, including alveolar bone lesions and ONFH (25). BMPs binding to BMP receptors to phosphorylate Smad, which is in turn translocated into the nuclei to activate osteogenic gene expression (26). Consistent with BMPs' osteoblatogenic function, BMP-2 overexpression promoted the number of osteoblasts in the nectoric femoral head in this study (Fig. 2C). In addition to osteogneic genes, BMPs also induce osteoblasts to express OPG, which blocks oetoclast production (27). In agreement with this reported result, BMP-2 transgenic ABMSCs recovered steroid-impaired OPG expression in this study (Fig. 5A and 5D). Thus, BMP-2 overexpression in ABMSCs has two functions. One is to facilitate ABMSC osteoblastogenesis, the other is to induce OPG expression to block osteoclastogenesis. These two functions enable BMP-2 transgenic ABMSCs greatly to ameliorate ONFH.

ABMSCs are located in alveolar bone, which is far from important organs and nerve system. AMBSCs are pluripotent stem cells and are able to differentiate into osteoblasts, chondrocytes and adipocytes (28). The method to isolate ABMSCs is much less invasive and easier than that to BMSCs. ABMSCs can be isolated and expanded from only 0.1–3 ml aspirate (29). All these features enable ABMSCs desirable for therapeutic application. In this study, ABMSCs exhibited a role to alleviate steroid-induced ONFH, and BMP-2 overexpression enhanced this capacity of ABMSCs.

In summary, ABMSCs and BMP-2 transgenic ABMSCs attenuated steroid-induced ONFH by promoting osteoblastogenesis and inhibiting osteoclastogenesis via blocking the RANKL/RANK/OPG pathway. Our results suggest that ABMSCs or transgenic ABMSCs are promising candidates for ONFH therapy.

ABMSCs and BMSCs have a similar ability to osteogenesis. ABMSCs are able to attenuate ONFH by promoting osteoblastogenesis and inhibiting osteoclastogenesis via inactivating the RANKL/RANK/OPG signaling pathway. These ABMSCs' functions are promoted by BMP-2 overexpression in ABMSCs.

Conflict of interest and funding

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