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Effect of ANGPTL7 on Proliferation and Differentiation of MC3T3-E1 Cells

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Background: Angiopoietin-like proteins (ANGPTL) are a family of secretory glycoproteins that are involved in many pathophysiological processes. ANGPTL7 is a newly-discovered member of the ANGPTL family and plays a role in corneal morphogenesis, angiogenesis, glaucoma, and cancer. To date, whether ANGPTL7 is involved in osteoporosis is unknown. Therefore, to discover the effects of ANGPTL7 on osteoporosis, we explored the expression of ANGPTL7 in preosteoblasts and assessed the mechanism underlying its effects on proliferation and differentiation abilities of preosteoblasts.

Material/Methods: Mouse MC3T3-E1 cells were cultured in osteogenic medium for osteogenic differentiation. The expression levels of ANGPTL7 were detected by RT-qPCR and Western blot assays. Moreover, the overexpressed plasmid of ANGPTL7 pMSCV-ANGPTL7 was transfected into MC3T3-E1 cells. CCK-8 was used to evaluate cell proliferation. ALP activity detection and alizarin red staining were performed to measure the effect of ANGPTL7 on osteogenic differentiation. The expression levels bone morphogenetic proteins (BMPs) and osteogenic markers ALP, runt-related transcription factor 2 (Runx2), osteocalcin (OCN), and collagen I (Col I) were analyzed by Western blot.

Results: When MC3T3-E1 cells were exposed to osteogenic medium, there was a significant increase in ANGPTL7, and overexpression of ANGPTL7 markedly promoted cell proliferation, ALP activity, and mineralization. Moreover, ANGPTL7 upregulated the levels of BMPs, especially BMP2/7, and the osteogenic markers ALP, Runx2, OCN, and Col I.

Conclusions: The results suggest that by regulating the expression of BMPs, ANGPTL7 directly promotes proliferation, differentiation, and mineralization of osteoblasts.

MeSH Keywords: Angiopoietins • Cell Dedifferentiation • Cell Proliferation • Osteoporosis

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Background

Osteoporosis is a skeletal system disease in which bones become fragile, fractures are caused by bone mass loss, and bone microstructure deteriorates. It has become a worldwide health problem that is increasingly attracting researchers' attention [1,2]. The causes of osteoporosis are very complex, including environmental, dietary, genetic, and other contributing factors. At present, the clinical treatments of osteoporosis, including estrogen replacement, promotion of bone formation, inhibition of bone resorption, and calcium supplementation, fail to achieve the desired results due to strong adverse effects and instability of the drugs [3,4]. Alternative natural treatments, which are more effective and have less adverse effects, are urgently needed. Previous research has revealed that the primary pathophysiological mechanism of osteoporosis is via impaired osteoblasts proliferation, differentiation, and mineralization [5,6]. Thus, research on osteoblasts is of great significance in understanding the mechanism of osteoporosis and in devising novel effective treatments.

The angiopoietin-like proteins (ANGPTLs) family, consists of 8 members (from ANGPTL1 to ANGPTL8), has homologous and similar domains with the angiopoietin family, and both contain an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain [7]. The role of angiopoietin in angiogenesis, reconstruction, and maintenance of normal bone structure and function has been widely recognized. ANGPTLs have the same structural domain as angiopoietin, and are also closely associated with angiogenesis [7,8]. In addition, accumulating evidence has revealed the important role of ANGPTLs in cell proliferation and differentiation. ANGPTL2 can inhibit the differentiation of pre-adipocytes and pre-osteoblasts, while overexpression of ANGPTL4 increases osteoclast differentiation and bone resorption. Both ANGPTL2 and ANGPTL4 are associated with osteoporosis [9,10]. Furthermore, ANGPTL7 can stimulate expansion of hematopoietic stem cells and progenitor cells. Previous studies have shown that ANGPTL7 is involved in a variety of physiological and pathological processes in the human body, such as corneal morphogenesis, angiogenesis, glaucoma, and cancer [11,12], but the role of ANGPTL7 in osteoporosis has not been previously reported. In this study, we investigated the role of ANGPTL7 in osteoporosis.

Material and Methods

Cell culture and transfection

The mouse pre-osteoblastic cell line MC3T3-E1 (ATCC; Manassas, VA, USA) was cultured in αMEM (Gibco, USA) supplemented with 10% FBS (Gibco, USA) in a humidified incubator (95% air, 5% CO_2) at 37°C. For osteogenic differentiation, cells were cultured with osteogenic medium (MUXMT-90021, Cyagen, Guangzhou, China) for 14 days, and the medium was replaced every 2 days.

For ANGPTL7 overexpression, the overexpressed plasmid with puromycin resistance of ANGPTL7 pMSCV- ANGPTL7 and the negative control pMSCV was designed and synthesized by Hanbio (Shanghai, China). The cells were plated in 6-well plates and transfection was conducted at 70–80% confluence. The retroviruses were packaged with 293FT cells, and the infected MC3T3-E1cells were selected with 2 μg/ml puromycin at 48 h after infection. Transfection used Opti-MEM, serum-free RMPI DMEM, and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The cells were cultured in 6-well plates under normal conditions and the culture medium was replaced every 2 days.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

MC3T3-E1 cells were harvested at 3, 7, and 14 days after osteogenic differentiation and after transfection of the cyclophilin B overexpression plasmid into MC3T3-E1 cells for 48 h. The total RNA was extracted using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Inc., USA) and the cDNA was synthesized with the RNA Transcription Kit (DRR037A, Takara Bio, Shiga, Japan). Then, PCR was performed using the SYBR Green RT-PCR Kit (Applied Biosystems, USA) on an ABI7500 RT-PCR instrument (ABI Company, Oyster Bay, NY, USA). GAPDH was used as the housekeeping gene, and the relative expressions of mRNAs were evaluated by the $2^{-\Delta\Delta Cq}$ method. The primers used were as follows:
ANGPTL7, Forward: 5'-TAAACGCAAGACACAGCTCAA-3',
Reverse: 5'-TGCATGATGTCAATCTGGTTGT-3';
GAPDH, Forward: 5'-TGGCCTTCCGTGTTCCTAC-3',
Reverse: 5'-GAGTTGCTGTTGAAGTCGCA-3'.

Cell Counting Kit-8 (CCK-8)

At 4 8h after transfection, MC3T3-E1 cells were seeded in 96-well plates at a density of 0.5×10^3 cells/ml and incubated for at 1, 3, and 5 days. Then, 10 μl of CCK-8 reagent (Roche Diagnostics, Basel, Switzerland) was added into every well. After 2 h of incubation, the optical density was measured at 490 nm using a microplate reader.

Western blot analysis

Total protein from the transfected MC3T3-E1 cells was extracted using RIPA lysis buffer (Millipore) and the concentration was assessed using a bicinchoninic acid (BCA) kit (BCA1-1KT, Sigma, St Louis, MO, USA). Protein samples (30 μg) were separated by 10% SDS-PAGE, and subsequently blotted onto a PVDF membrane (EMD Millipore, Billerica, MA, USA). After incubation at 4°C overnight with specific primary antibodies, the blot

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was probed with corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Then, the immunoreactive bands were visualized with the ECL detection reagent (Millipore). GAPDH was used as the internal loading control. The antibodies used were: ALP (1: 1000, Cell Signaling Technology, Inc.), OCN (1: 1000, Cell Signaling Technology, Inc.), Runx2 (1: 1000, Cell Signaling Technology, Inc.), Collagen1 (1: 1000, Cell Signaling Technology, Inc.), BMP2 (1: 1000, Millipore), BMP4 (1: 1000, Millipore), BMP6 (1: 1000, Millipore), BMP7 (1: 1000, Millipore), mouse anti-rabbit secondary antibody (1: 10 000, Santa Cruz, sc-2357), and rabbit anti-mouse secondary antibody (1: 10 000, Santa Cruz, sc-358914).

Alkaline phosphatase (ALP) activity assay

At 7 days after transfection, MC3T3-E1 cells were harvested and lysed with 0.2% Triton X-100, and then were tested with the Alkaline Phosphatase Diethanolamine Activity Kit (AP0100, Sigma, St. Louis MO, USA) according to the manufacturer's protocols. Protein concentration was measured using a BCA kit (Sigma, St Louis, MO, USA), and the relative ALP activity was normalized to the protein concentration.

Mineralization assay

Alizarin red-S staining was used to measure the calcium deposition. Briefly, at 21 days after transfection, MC3T3-E1 cells were fixed with 4% paraformaldehyde for 30 min. Then, cells were stained with 40 mM alizarin red-S (BS0430, BIOSHARP, USA) for 30 min at 37°C. After washing with distilled water to remove the unbound dye, the mineralized nodules were photographed. To quantify matrix mineralization, the calcium-bound alizarin red was dissolved and released with 10% (v/v) acetic acid for 30 min at room temperature. Subsequently, the supernatant is collected and 10% (v/v) ammonium hydroxide was added. Absorbance of the solution was measured at 405 nm.

Statistical analysis

All data are expressed as the mean±standard deviation (SD). Statistical differences between the groups were assessed by one-way ANOVA statistical analysis followed by a Tukey's test if necessary. All experiments were repeated at least 3 times, and $p<0.05$ was considered significant.

Results

Detection of ANGPTL7 expression in osteogenic medium-induced MC3T3-E1 cells

To detect the expression of ANGPTL7 during osteoblast differentiation, MC3T3-E1 cells were cultured in osteogenic medium for 3, 7, and 14 days. The results of Western blot analysis showed that the expression of ANGPTL7 increased, reaching the highest level at 7 days and continued to be highly expressed until 14 days in osteogenesis-induced MC3T3-E1 cells (Figure 1A). RT-qPCR assay showed similar results to those of Western blot (Figure 1B). To further investigate the role of ANGPTL7 in osteogenic differentiation, pMSCV- ANGPTL7, the overexpressed plasmid of ANGPTL7, was transfected into MC3T3-E1 cells. At 48 h after transfection, the transfection efficiency was assessed. As showed in Figure 1C and 1D, ANGPTL7 expression was significantly increased at both protein and mRNA levels in pMSCV-ANGPTL7-transfected MC3T3-E1 cells.

Effect of ANGPTL7 on the proliferation of MC3T3-E1 cells

To determine the effect ANGPTL7 on the proliferation of MC3T3-E1 cells, CCK-8 assay was performed at 48 h after transfection. As shown in Figure 2A, ANGPTL7 overexpression markedly increased cell proliferation of MC3T3-E1 cells at 1, 3, and 5 days.

Effect of ANGPTL7 on the differentiation and mineralization of MC3T3-E1 cells

At 7 days after transfection, the ALP activity was assessed, and the results suggested that ANGPTL7 significantly increased ALP activity compared with control and NC groups (Figure 2B). Moreover, the expression levels of the osteoblast differentiation markers ALP, Runx2, OCN, and Col I were measured by Western blot, showing that overexpression of ANGPTL7 significantly increased ALP, Runx2, OCN, and Col I expression (Figure 3). In addition, the effect of ANGPTL7I on mineralization was determined using alizarin red-S staining to measure the deposition at 21 days after transfection. The formation of mineralized bone nodules is an important marker of osteoblast maturation. As shown in Figure 4, compared with the NC group, much more calcium deposition was found in pMSCV-ANGPTL7-transfected MC3T3-E1 cells. The activity of mineralization osteogenesis in the overexpressed ANGPTL7 group was increased compared with the control group and the NC group.

Effect of ANGPTL7 on BMPs expression in MC3T3-E1 Cells

BMPs are the key regulators in osteoblast differentiation and have been approved for use in clinical practice. Therefore, the expression levels of BMP2, 4, 6, and 7 were examined using Western blot assay at 7 days after transfection. The results revealed a significant increase in BMPs induced by ANGPTL7, among which the most significant effect was observed in BMP2/7 (Figure 5).

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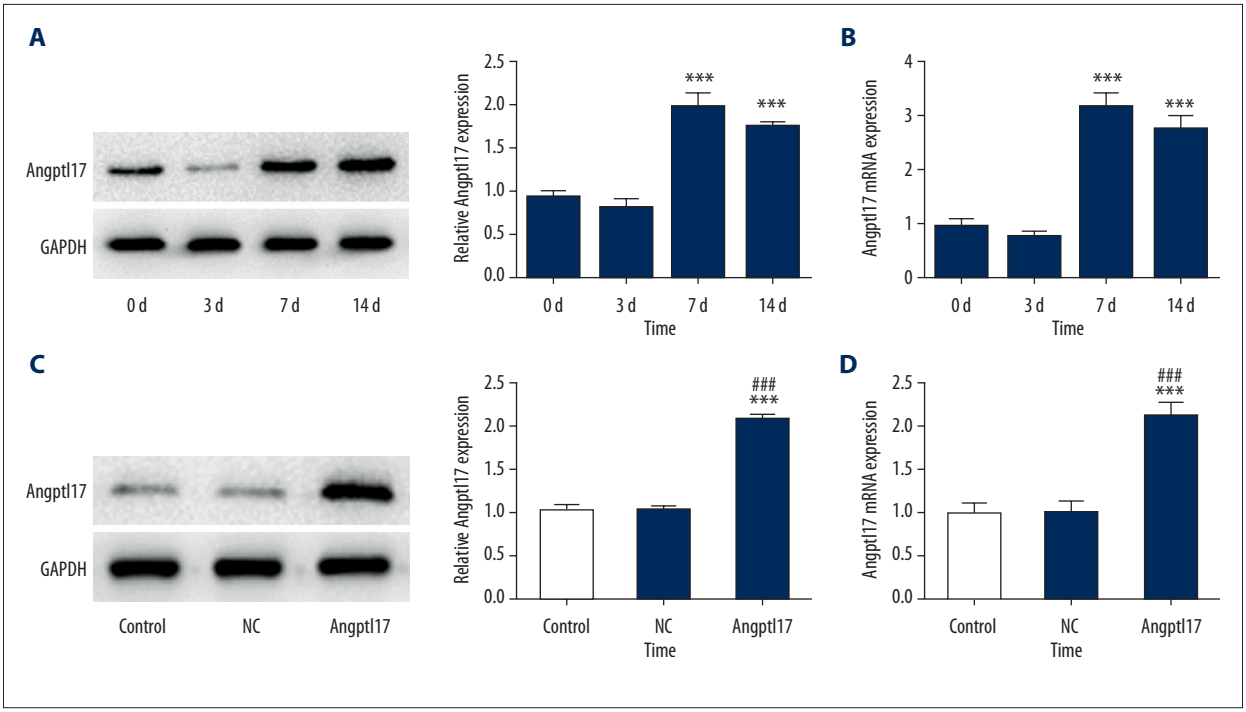

Figure 1. MC3T3-E1 cells were cultured in osteogenic medium for 0, 3, 7, and 14 days. Western blot (**A**) and RT-qPCR (**B**) assays were performed to measure the expression levels of *ANGPTL7*. The overexpressed plasmid of ANGPTL7 pMSCV-ANGPTL7 was transfected into MC3T3-E1 cells. At 48 h after transfection, Western blot (**C**) and RT-qPCR (**D**) assays were performed to detect the expression levels of *ANGPTL7*. The data shown are representative of 3 individual experiments. *** P<0.001 *vs.* 0 day or Control groups; ### P<0.001 *vs.* NC group.

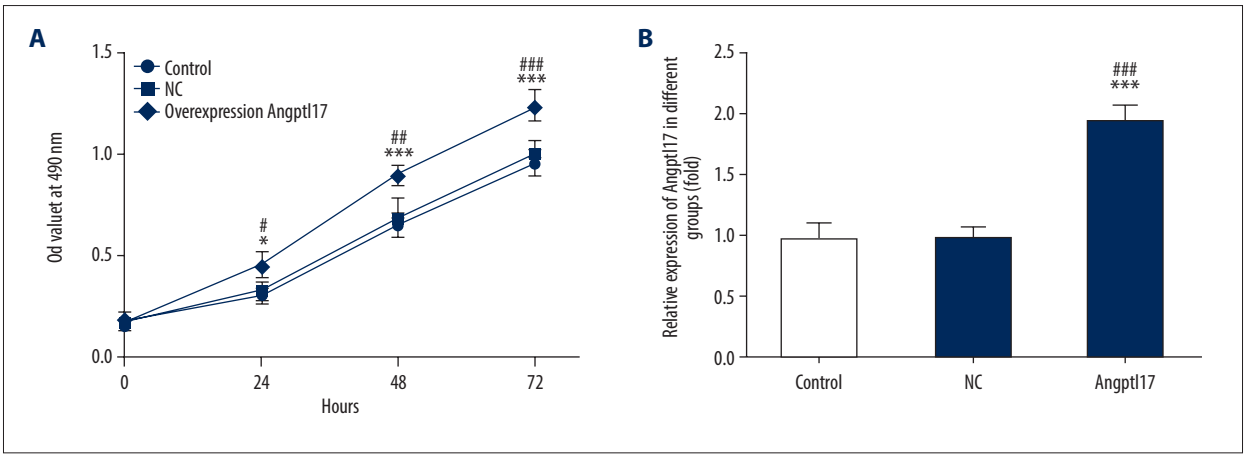

Figure 2. The overexpressed plasmid of ANGPTL7 pMSCV-ANGPTL7 was transfected into MC3T3-E1 cells. At 48 h after transfection, CCK-8 assay (**A**) was performed to assess cell proliferation and ALP activity (**B**) was determined. The data shown are representative of 3 individual experiments. * P<0.05, *** P<0.001 *vs.* Control group; # P<0.05, ## P<0.005, ### P<0.001 *vs.* NC group.

Discussion

Osteoporosis is a systemic and multifactorial disease that can occur in people of both sexes and at any age, but it is more common in postmenopausal women and older men [1,13]. Current clinical treatment has not achieved the desired effect due to adverse effects and instability of drugs [14,15]. Therefore, many researchers are devoted to developing new drug targets and therapies for treatment of osteoporosis. The main finding of our study is that ANGPTL7 is a powerful regulator that plays a crucial role in promoting cell proliferation and differentiation by upregulating BMPs, which provides a new insight into

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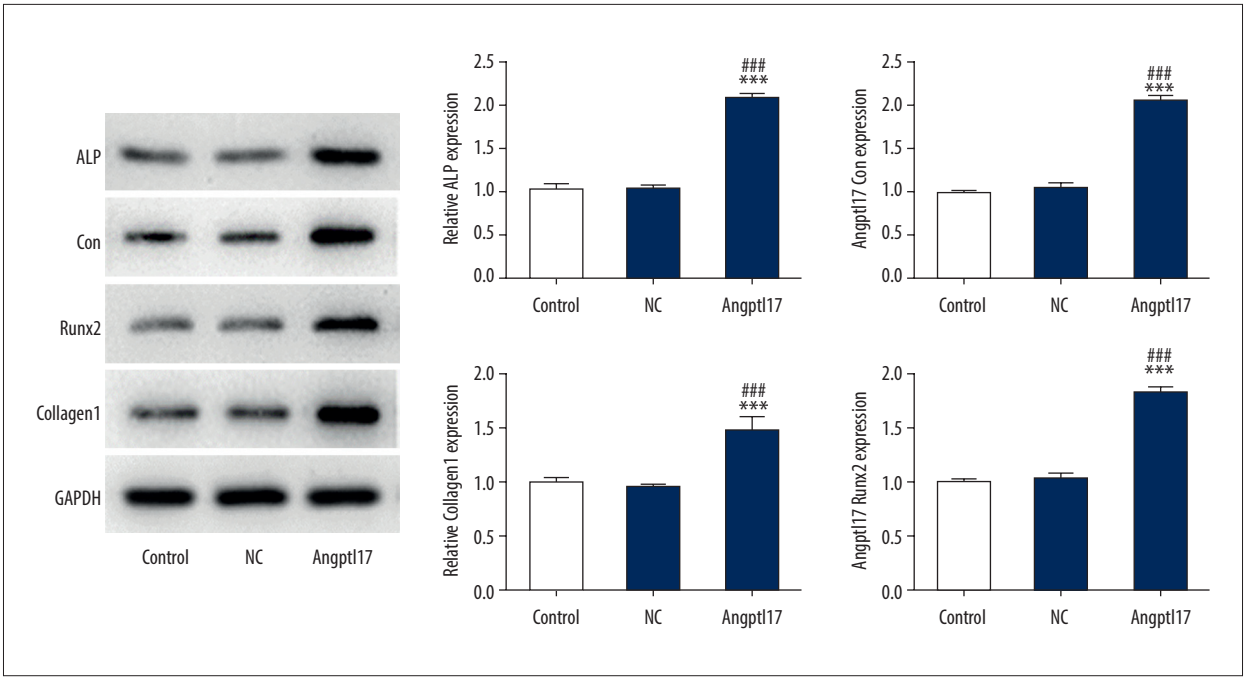

Figure 3. The overexpressed plasmid of ANGPTL7 pMSCV-ANGPTL7 was transfected into MC3T3-E1 cells. At 7 days after transfection, the ALP, Runx2, OCN, and Col I protein expression levels were measured by Western blot assay. The data shown are representative of 3 individual experiments. *** P<0.001 *vs.* Control group; ### P<0.001 *vs.* NC group.

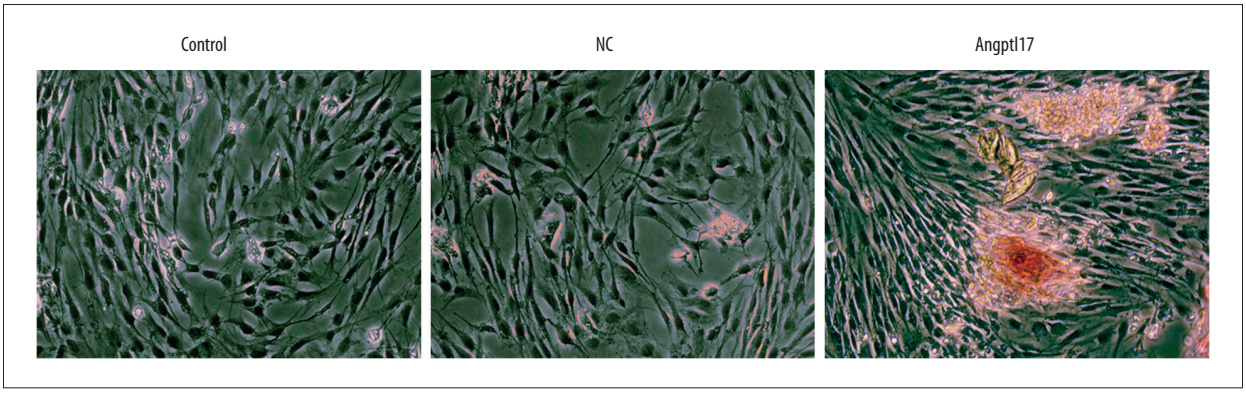

Figure 4. The overexpressed plasmid of ANGPTL7 pMSCV-ANGPTL7 was transfected into MC3T3-E1 cells. At 21 days after transfection, the deposition was measured by alizarin red-S staining.

the mechanism of osteoporosis and shows that it is a potential drug target for osteoporosis treatment.

ANGPTL 7 protein exists in aqueous humor of normal people, but is highly expressed in aqueous humor of glaucoma patients. ANGPTL 7 was also found to be highly expressed in colon, breast, and ovarian cancers [11]. ANGPTL 7 significantly increased the phosphorylation of Erk, Akt, and FAK kinases, which are associated with proliferation, survival, and migration of vascular endothelial cells [12]. However, there has been no relevant research on ANGPTL 7 in bone tissue. The function of ANGPTLs in angiogenesis has been widely accepted, but the differential expression of ANGPTLs in various tissues and cells suggests it has diverse functions. A growing body of research suggests that members of the ANGPTL family have pro-inflammatory and tumor-suppressing functions [7]. It has been reported that the expression of ANGPTL4 was significantly increased in rats with diabetic retinopathy induced by high glucose, and further studies showed that ANGPTL4 enhanced the secretion of inflammatory factors through activating Profilin-1 [16]. Similarly, the level of ANGPTL2 was significantly increased in *Porphyromonas gingivalis* lipopolysaccharide (LPS)-treated human gingival epithelial cells, and recombinant human ANGPTL2 strongly induced the expression of pro-inflammatory factors such as TNF-α, IL-1β, and IL-8 [17]. Moreover, knockdown of ANGPTL2 markedly suppresses cell

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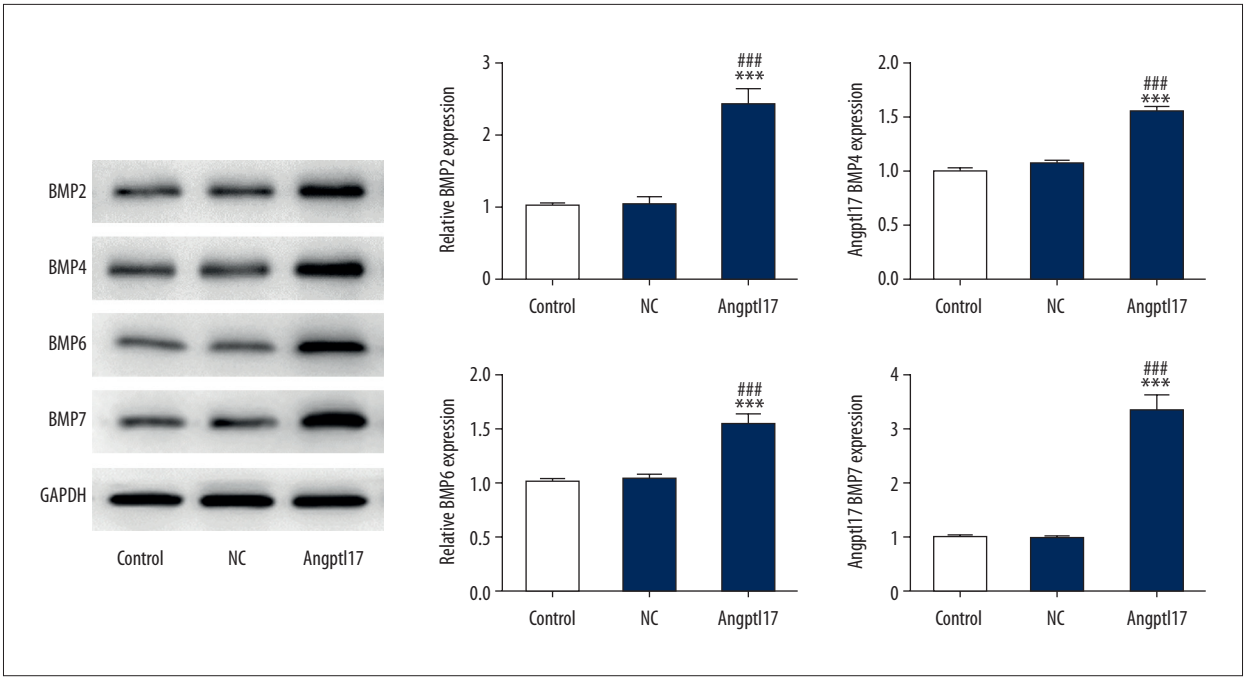

Figure 5. The overexpressed plasmid of ANGPTL7 pMSCV-ANGPTL7 was transfected into MC3T3-E1 cells. At 7 days after transfection, the expression levels of BMP2, 4, 6, and 7 were examined using Western blot assay. The data shown are representative of 3 individual experiments. *** P<0.001 *vs.* Control group; ### P<0.001 *vs.* NC group.

proliferation, migration, and invasion in human prostate cancer cells [18]. These studies demonstrate the potential of multiple physiological functions of ANGPTLs in different tissues and cells. Importantly, Li et al. have shown that ANGPTL2 silencing inhibited macrophage colony-stimulating factor-induced proliferation and pro-inflammatory cytokines expression via NF-κB/MAPKs/Cyclin pathways in osteoclast precursor cells [19]. The present study found that overexpression of ANGPTL7 significantly promoted cell proliferation and differentiation by modulating the BMPs pathway in the mouse pre-osteoblastic cell line MC3T3-E1, providing new insights into understanding the mechanism underlying the physiological function of ANGPTLs in the skeletal system.

In this study, the results suggested that overexpression of ANGPTL7 significantly increased ALP, OCN, Runx2, and Col I expression. ALP activity assay and alizarin red-S staining were used to determine the effect of ANGPTL7 on osteoblast differentiation and mineralization. ALP alkaline phosphatase is enriched in the cytoplasm of osteoblasts and is one of the functional enzymes that reflect the viability of osteoblasts. It has very important physiological significance for bone formation. Runx2, a transcription factor at the downstream end of bone morphogenetic protein (BMP) signaling pathways, is essential for osteoblast differentiation and bone formation. The expression of these osteogenesis-related genes promotes osteoblast-related functions such as proliferation, differentiation, and mineralization of rat osteoblasts *in vitro*. It is generally believed that the expression levels of Col1a1, ALP, OCN, and other genes are reliable indicators for the differentiation of osteoblasts and mineralization [20], and overexpression of ANGPTL7 increases differentiation of MC3T3-E1 cells.

BMPs are well known as a class of strong osteoinductive factors that are considered to play a major role in osteogenic differentiation [21,22]. In recent years, the osteogenic function of BMPs has been extensively studied, confirming that BMPs play a key role in the stable development of many parts of the skeletal muscle system [23]. At present, it is generally believed that inhibition of the BMP pathway in osteoblasts and osteoclasts can enhance bone resorption by regulating osteoclasts formation, resulting in increased bone mass [24]. The promotion of bone mass production by the BMP pathway is closely related to chondrocytes. In particular, BMP2, 4, 6, and 7 have been used in a number of clinical trials due to their great potential in bone healing [25–27]. BMP2 is an inducing factor of bone formation, which can induce mesenchymal cells to proliferate and differentiate into osteoblasts or chondrocytes. BMP2 plays an important role in bone formation, induction, and repair. BMP2 can directly or indirectly participate in the formation, differentiation, maturation, and activity of osteoclasts. BMP4 is a key osteoblast factor that is related to the early pathological changes of heterotopic ossification and can stimulate the expression of alkaline phosphatase, type I collagen, and osteocalcin. ANGPTL4 appears to promote metastasis and inhibit apoptosis of colorectal cancer cells by upregulation

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of BMP7. We examined the expression levels of BMP2, 4, 6, and 7 using Western blot analysis at 7 days after transfection. The results suggested that upregulation of ANGPTL7 significantly induced the expression of BMPs, especially BMP2/7. The results revealed that BMP signaling pathways were involved in ANGPTL7-induced proliferation and differentiation of MC3T3-E1 cells.

Overall, the present investigation revealed that the expression level of ANGPTL7 was increased in osteogenic medium-induced MC3T3-E1 cells. We also found that overexpression of ANGPTL7 promoted MC3T3-E1 proliferation, differentiation, and mineralization of MC3T3-E1 cells via the BMPs pathway. Taken together, these findings indicated that ANGPTL7 is a potent osteogenic differentiation-inducing factor that also promotes BMPs expression, suggesting its potential in the treatment of osteoporosis. Of course, this experimental study has certain limitations and shortcomings. This study was based on *in vitro* cell levels and only mouse MC3T3-E1 cells were used in this study. In further studies, we will focus on the role of ANGPTL7 in other cell lines and animal models.

Conclusions

In summary, the results of this study suggest that ANGPTL7 can directly promote proliferation, differentiation, and mineralization of osteoblasts by regulating the expression of BMPs.

Conflict of interests

None.

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