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Original Article

Msx2 plays an important role in BMP6-induced osteogenic differentiation of two mesenchymal cell lines: C3H10T1/2 and C2C12

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ABSTRACT

Bone morphogenetic proteins (BMPs), have been shown to enhance the osteogenic differentiation of mesenchymal cells (MCs) and to promote bone formation. BMP6 is known to play an important role in the process of MCs towards osteogenic differentiation by virtue of their osteoinductive and cell type specific proliferative activity. However, the molecular mechanism relate to BMP6 osteoinductive activity is still unclear and continues to warrant further investigation. Msx2 is a member of the homeobox gene family of transcription factors and promotes calcification. Hence, we wondered if it might also play a role in BMP6-induced osteogenesis. In this study, two mouse mesenchymal cell lines were treated with BMP6, adenovirus-Msx2 (Ad-Msx2) or adenovirus-siMsx2 (Ad-siMsx2). Based on the results of mRNA and protein expression, it was indicated that BMP6 could enhance the expression of Msx2 and activate the phosphorylation of Smad 1/5/8, p38 and ERK1/2. Being transfected by Ad-Msx2, the BMP6-induced activation of phosphorylation was significantly promoted. On the contrary, two cell lines transfected by Ad-siMsx2 presented an inhibited expression of three phosphorylated proteins even after being induced by BMP6. The evaluation of ALP, OPN, OC and calcium deposits revealed the osteogenic results those were corresponding to the results of mRNA and protein. Taken together, these findings can be a novel viewpoint for the understanding of the mechanisms of BMP6-induced osteogenesis and provide therapeutic targets of bone defect.

1. Introduction

Bone tissue defect is a common issue in clinic. Although bone tissue has some extent inherent ability of regeneration, there are still 5–10% of fracture patients facing insufficient healing [1]. As the "gold standard" method for injured bone repairment, autologous bone graft has been widely used in clinic. Nevertheless, the donor-site morbidity is still an unsolved issue in the process [2,3]. Tissue engineering is a promising tool for bone tissue reconstruction. Two mouse mesenchymal cell lines (MCs), C3H10T1/2 and C2C12, were widely used in studies of osteogenic differentiation and bone tissue engineering [4,5]. Due to the similar features with tissue-derived mesenchymal stem cells (MSCs), the study of these two cell lines may provide us with a lot of useful information.

Bone morphogenetic proteins (BMPs), the largest subdivision of transforming growth factor-β (TGF-β) superfamily, contain 20 identified members and play a key role in the development and homeostasis of organs [6,7]. Osteogenesis is the most studied biological function of BMPs. After regulating the BMPs signaling antagonists and agonists, the adipose-derived stem cells (ASCs) display a significant up-regulated osteogenic differentiation [8]. The poly lactic acid (PLA) scaffold combined with BMP2 has been proved to have a potential for promoting the osteogenic differentiation of MSCs [9]. Similarly, during the repairing process of rat calvarial defect with a 3D vehicle system that contains MSCs and BMP2, the enhanced osteogenic differentiation and new bone formation have been observed [10]. For a long time, BMP2 has been considered as the most important member in BMPs' family which is associated with osteogenesis. Interestingly, recent studies have indicated that BMP6 may have a stronger effect of osteogenesis than BMP2 [1,11]. BMP6 has

Abbreviations: BMP, Bone morphogenetic protein; Msx, Msh homeobox; Ad, adenovirus-transfection; siRNA, silencing RNA.

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been demonstrated to possess a potential for restoring the osteogenesis capability of the bone marrow mesenchymal stem cells derived from the type I diabetes [12]. The calcium phosphate scaffold combined with BMP6 greatly facilitated the osteogenesis of human periosteum derived progenitor cells and new bone formation [13]. However, the molecular mechanism relates to BMP6-induced osteogenesis is still unclear and needs a further investigation.

As a member of the homeobox gene family of transcription factors, Msh homeobox 2 (Msx2) is highly expressed in the axial skeleton and is required for craniofacial, tooth and limb development. It has been regarded as a key factor in vascular calcification [14]. Moreover, there is a synergy effect between Msx2 and BMP2 on osteogenic differentiation [15]. A study focused on the effect of overexpressed microRNA-203 on osteogenic differentiation of osteoblast revealed that this promoted osteogenesis was induced by up-regulated Msx2 [16]. These results indicate that Msx2 play a key role in osteogenesis and associate with BMPs during the calcification process. Hence, we hypothesized that Msx2 may also play a key role in BMP6-induced osteogenic differentiation.

2. Methods

2.1. Cell culture and recombinant BMP6 treatment

Two types of mouse mesenchymal cell lines including C3H10T1/2 (ATCC, CRL-3268) and C2C12 (ATCC, CRL-1772), were cultured on 6-well plastic plates with a concentration of 1.0×10^5 cells/well. 2 mL DMEM containing 10% FBS was added into each well and replaced for every other day. After reaching 80–90% confluence of all samples, 200 ng/mL recombinant BMP6 was added into each well. Cells cultured with DMEM were served as blank.

2.2. BMP6 induction

For evaluating the effect of BMP6 on Msx2 expression in MCs, the expression levels of mRNA and protein were detected by quantitative real-time PCR (qRT-PCR) and Western blot, respectively. After being treated by BMP6 for 24 h, the total RNA of each sample was harvested by TRIzol Reagent (Invitrogen, USA). A reverse transcription kit (Takara, Japan) was used to synthesize the cDNA of all samples. All primers were designed by Life Technologies (ThermoFisher Scientific, USA) and the sequences of primers were displayed in Table 1. The quantification of mRNA level of Msx2 was performed using a SYBR green Supermix (Takara, Japan) on a Roche Lightcycler 96 system (Roche, Switzerland). All data were normalized by glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and analyzed using the $2^{-\triangle\triangle Ct}$ method. Total proteins of each sample were extracted from the organic phase after the TRIzol treatment and dissolved in 1% SDS. After being mixed with 2 × loading buffer (Beyotime Biotechnology, China) and heated at 100 °C for 5–10 min, all specimens were separated by 10% Tris-SDS-PAGE.

Table 1
Primer sequences and product sizes used for quantitative real-time PCR.

Genes	5′-3′	Primer sequences	Production size (bp)
Msx2	Forward	CTGGTGAAGCCCTTCGAGAC	133
	Reverse	ATATGTCCTCCTACTCCTGCCC	
GAPDH	Forward	GCAAGTTCAACGGCACAG	140
	Reverse	GCCAGTAGACTCCACGACAT	
Dlx5	Forward	CTACCAGTACCAGTACCACGG	148
	Reverse	TTCTTTCTCTGGCTGGCTGGT	
Osx	Forward	AGTGGGAACAAGAGTGAGCTG	145
	Reverse	TAGGAGCTTCTTCCTGGGT	
Runx2	Forward	CCCAGTATGAGAGTAGGTGTCC	149
	Reverse	GGGTAAGACTGGTCATAGGACC	

All separated proteins were transferred onto 0.22 μm PVDF membrane by wet-blotting system (Bio-Rad, USA). After being blocked with 5% skim milk, PVDF membrane was incubated with primary antibody (Rabbit anti-Msx2, Abcam, ab223692; Rabbit anti-Gapdh, Abcam, ab181602) and horseradish peroxidase conjugated secondary antibody (Goat anti-Rabbit, Abcam, ab223692) in sequence. The target blot was observed using a CCD camera gel imaging system (ChemiDoc XRS, Bio-Rad, USA).

2.3. Overexpression and attenuation of Msx2

The sequence of mouse full-length Msx2 gene was obtained from GenBank. The adenovirus that encoding Msx2 was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The MCs were transfected with adenovirus encoding GFP or Msx2 at MOI of 50:1 in DMEM. After 24 h, the viral particles were discarded. Cells were harvested for extracting the total proteins. The proteic expressions of Msx2 in C3H10T1/2 and C2C12 were detected by Western blot assay.

The process of Msx2 attenuation was performed according to previous study [17]. The sequences of siRNA for Msx2 were 5′-GCAGGCAGCGUCCAUAUAUTT-3′ and 5′-UAUACAUUGCGCGCGUGUUU-3'. Briefly, 50 nmol siRNA was mixed with SilentFect™ Reagent (Hercules, CA). Then the mixture was supplemented into the 6-well culture plates when two types of MCs were 70% confluence. After 24 h incubation, the total proteins of each sample were extracted. Western blot assay was used to evaluate the proteic expression of Msx2 in C3H10T1/2 and C2C12.

2.4. Detection of early and late osteogenesis in C3H10T1/2 and C2C12

In this work, 6 groups including MCs (blank), Ad-Msx2-transfected MCs (Ad-Msx2), siRNA-transfected MCs (siMsx2), BMP6-induced MCs (BMP6), Ad-Msx2-transfected-BMP6-induced MCs (BMP6+Ad-Msx2), and siRNA-transfected-BMP6-induced MCs (BMP6+siMsx2) were prepared. For the detection of early stage osteogenesis in each group, the expressions of ALP and two bone-related proteins (osteopontin, OPN and osteocalcin, OC) were measured after 7 d culture. The relative activity of ALP in each group was detected with the same protocol in previous study [18]. Briefly, all samples were washed with PBS and lysed by sonification. Then the lysates were incubated with p-nitrophenol phosphate (Sigma, USA) at 37 °C for 1 h 1 M sodium hydroxide was used to stop the enzymatic reaction. The absorbance was measured at 540 nm (Biotek, USA).

At the end of 7 d culture, the total proteins of each group were harvested and separated by SDS-PAGE. After transferring, blocking, incubating with primary antibodies (rabbit anti-OPN, Abcam, ab8448; rabbit anti-OC, Abcam, ab93876) and secondary antibody (Goat anti-Rabbit, Abcam, ab223692), the target bands were observed using a CCD camera gel imaging system (ChemiDoc XRS, Bio-Rad, USA).

Calcium deposits were regarded to be the late stage marker of osteogenesis. In this study, after 21 d culture, all samples from two MCs were washed with PBS and fixed with 4% paraformaldehyde for 10 min. After thrice washing with distill water, all samples were stained by 0.2% alizarin red reagent (Solarbio, China) for 30 min. Then each sample was washed by distill water and observed by microscope (Olympus, Japan). Orange red indicates the positive staining of calcium deposits.

2.5. Detection of pivotal osteogenic transcription factors

After 24 h culture, C3H10T1/2 (6 groups) in all six groups were harvested and extracted the total RNA and proteins. The genetic

and proteic expression levels of runt-related transcription factor 2 (Runx2), osterix (Osx) and distal-less homeobox 5 (Dlx5) were detected in this work. The specific primers were shown in Table 1. The procedures of qRT-PCR and Western blot are same as described before. Primary antibodies involved in this work were rabbit anti-Runx2 (Abcam, ab192256), rabbit anti-Osx (Abcam, ab209484) and rabbit anti-Dlx5 (Abcam, ab109737). The following second antibody was goat anti-rabbit (Abcam, ab223692).

2.6. Activation of signaling pathway

The total proteins of C3H10T1/2 (6 groups) were also used to detect the proteic expressions of p-Smad 1/5/8, Smad 1/5/8, p-p38, p38, p-ERK 1/2 and ERK 1/2. Primary antibodies involved in this experiment were rabbit anti-p-Smad 1/5/8 (CST, 13820), rabbit anti-Smad 1/5/8 (Abcam, ab13723), rabbit anti-p-p38 (Abcam, ab4822), rabbit anti-p38 (Abcam, ab170099), rabbit anti-p-ERK 1/2 (Santa Cruz, sc-136521), and rabbit anti-ERK 1/2 (Abcam, ab17942). The following second antibody was goat anti-rabbit (Abcam, ab223692).

2.7. Statistical analysis

In this study, all experiments were repeated at least three times. Data presented in this manuscript are from one independent set. The statistical analysis was performed by *t*-test and ANOVA. All data were presented as means ± standard error. $P < 0.05$ indicated that the difference is significant.

3. Results

3.1. qRT-PCR and Western blot of Msx2 after treating with BMP6, Ad-Msx2 and Ad-siMsx2

After treating with BMP6 for 24 h, both of C3H10T1/2 and C2C12 showed a significantly higher mRNA expression level of Msx2 ($p < 0.001$) than those in blank control. Similarly, the significantly up-regulated levels of Msx2 protein ($p < 0.001$) were also observed in BMP6 groups (Fig. 1A). After transfection with Ad-Msx2, the expressions of Msx2 protein in both two MCs were significantly increased. On the contrary, the expressions of Msx2 protein in two MCs were significantly decreased after attenuation by Ad-siMsx2 (Fig. 1A). These results showed that either supplementation of BMP6 or overexpression of Msx2 could significantly improve the expression of Msx2 in both types of MCs. After silencing, the expression of Msx2 can be effectively inhibited. In addition, the Ad-GFP group showed no difference with blank group. This indicated that no effect of transfected-GFP on the expression of Msx2 in both two MCs.

3.2. ALP activity

After 7 d cultivation, there was no difference ALP activity in C3H10T1/2 among blank group, Ad-Msx2 group and Ad-siMsx2 group. The ALP activity in other three groups including BMP6, BMP6+Ad-Msx2, and BMP6+Ad-siMsx2 were significantly higher than that in blank group. BMP6+Ad-Msx2 group was the highest one, followed by BMP6 group and BMP6+Ad-siMsx2 group. There were significant differences among these three groups. Interestingly, the results of ALP activity in C2C12 displayed a similar trend with that in C3H10T1/2 (Fig. 2 A).

3.3. Western blot of OPN and OC

twAfter 7 d cultivation, the expression of OPN was similar in two types of MCs. In the order of expression level of OPN from high to low, each group was BMP6+Ad-Msx2, BMP6, Ad-Msx2, BMP6+Ad-siMsx2, and blank, respectively. There were differences between two MCs in the expression of OC. In C3H10T1/2, the expression of OC of blank and Ad-siMsx2 were almost negative. The BMP6+Ad-Msx2 group presented the highest expression of OC, followed by BMP6, BMP6+Ad-siMsx2, and Ad-Msx2. Nevertheless, all groups in C2C12 displayed positive expression of OC. As the two groups with the highest expression levels of OC, there was almost no difference between BMP6+Ad-Msx2 group and BMP6 group. In the remaining groups, the expression levels from high to low were Ad-Msx2, blank, BMP6+Ad-siMsx2, and Ad-siMsx2 (Fig. 2 B).

3.4. Calcium deposits staining

The most positive staining of calcium deposits was observed in BMP6+Ad-Msx2 group for both MCs. Two BMP6 groups in both MCs displayed the second most positive staining, followed by two

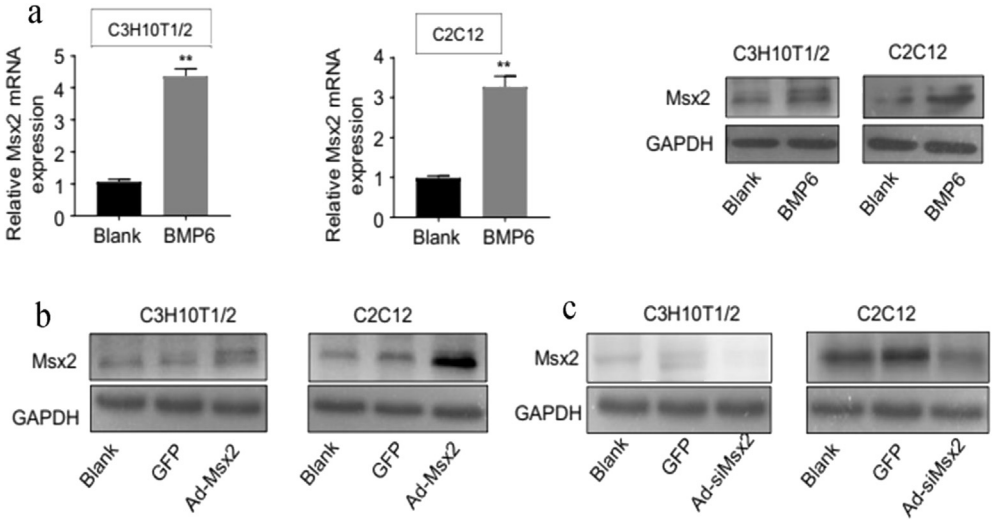

Fig. 1. The expression of Msx2 in two MCs after treating with BMP6, Ad-Msx2 and Ad-siMsx2. The mRNA and protein expression of Msx2 after BMP6 induction (A). Western blot of Msx2 in two MCs after overexpression or silencing (B and C).

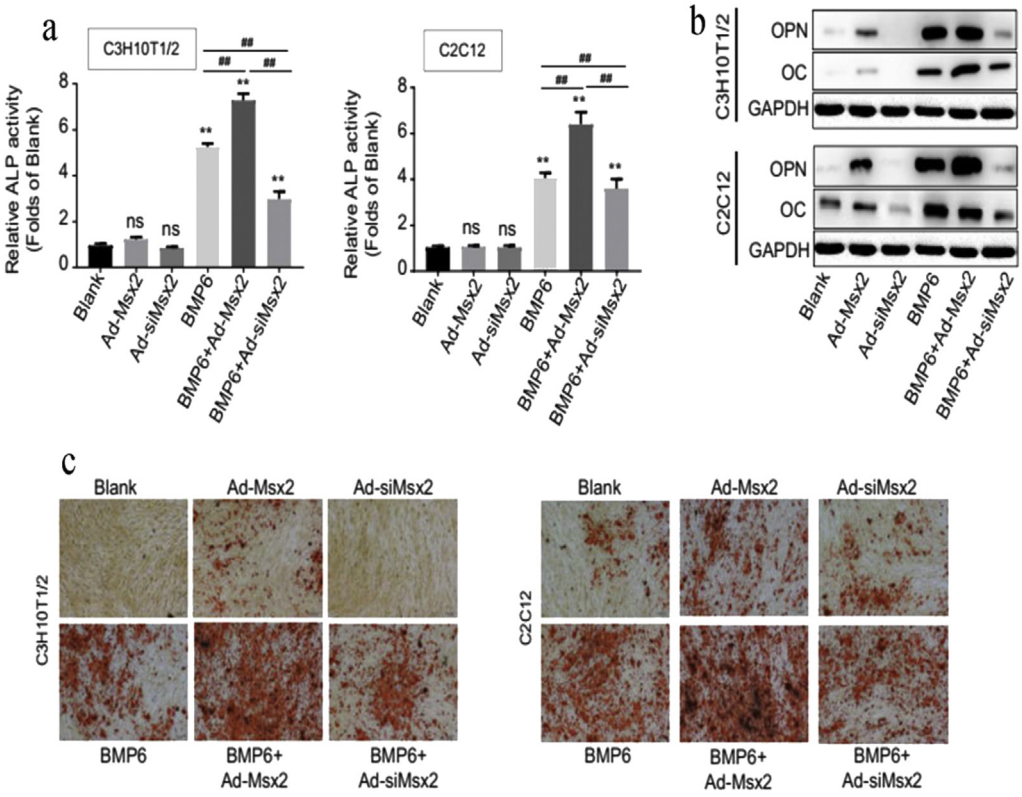

Fig. 2. Early and Late osteogenesis of MCs. The relative ALP activity of two MCs in 6 groups (A). The expression of two key proteins (B). The staining of calcium deposits in each group (C). * indicates $p < 0.05$, compared with blank. # indicates $p < 0.05$ between two groups.

BMP6+Ad-siMsx2 groups and two Ad-Msx2 groups. The significant difference between two MCs was mainly in blank group and Ad-siMsx2 group. In C3H10T1/2, almost no calcium deposits of blank group and Ad-siMsx2 group was stained. However, in C2C12, there was a small amount of positive staining of calcium deposits in these two groups (Fig. 2C).

3.5. Osteogenic differentiation

The significantly increased expressions of Runx2, Osx and Dlx5 were observed in BMP6+Ad-Msx2 group, compared with other 5 groups. In addition to BMP6+Ad-Msx2 group, BMP6 group displayed highest expression of three genes among 5 groups. Three genes expression in BMP6+Ad-siMsx2 group was rank second only to group BMP6 (Fig. 3A). Western blot assay also indicated that BMP6+Ad-Msx2 group possessed the highest expression of Msx2 protein. It was also followed by BMP6 group and BMP6+Ad-siMsx2 group (Fig. 3B).

3.6. Activating of signaling pathway

Fig. 4 presented the expressions of proteins which are involved in osteogenesis-related signaling pathways. The expression of unphosphorylated proteins including Smad 1/5/8, p38 and ERK 1/2 was no significant different in each group, whilst the expression of phosphorylated proteins was quite different. Intriguingly, the expressions of three phosphorylated proteins were still the highest in BMP6+Ad-Msx2 group. This was consistent with the results of previous assays. These results indicated that BMP6 could effectively activate the phosphorylation of Smad 1/5/8, p38 and ERK 1/2. Msx2 further enhances this activation. Silencing Msx2 could impair the phosphorylation that was activated by BMP6.

4. Discussion

As an important component of cell-therapy strategy, exogenous cells are widely used to repair bone defects [19,20]. These cells must be exposed to a large number of growth factors when they are working in the niche of bone regeneration. BMPs are the most remarkable growth factors that are associated with bone healing [21]. BMP6 is the one of 20 members of BMPs family and it is demonstrated to have a great potential in promoting the bone regeneration in recent studies [22,23]. It is worth noting that BMP6 is capable of inducing bone growth at a lower dose than other BMPs [1,24,25]. It may have a greater advantage in bone defect repair. Therefore, this study focused on the molecular mechanism of BMP6-induced osteogenesis.

Msx2, a member of homeobox gene family of transcription factors, has involved in the vascular calcification [26]. It also plays an important role in BMP2-induced osteogenesis [27,28]. The overexpressed Msx2 could enhance the osteogenesis of mouse blastema-like cells via activation of BMP4 [29]. Hence, it may be noteworthy in BMP6-induced osteogenesis. In the present study, we first examined the expression of Msx2 in two MCs after BMP6-inducing. Similar with previous studies, the mRNA and protein expression of Msx2 was significantly up-regulated after BMP6-inducing [30,31]. Interestingly, previous studies indicated that BMP4 up-regulated the expression of Msx2 by activating Smad 1/5 signaling pathway [32,33]. In this work, we also observed that the expression of phosphorylated Smad 1/5/8 in BMP6 group was higher than that in blank group. This phenomenon suggested that BMP6 may also regulate Msx2 expression through Smad signaling pathway, but the specific relationship between them needs further study. In addition, we also detected the expression of Msx2 in MCs after overexpression and attenuation of Msx2. The results indicated

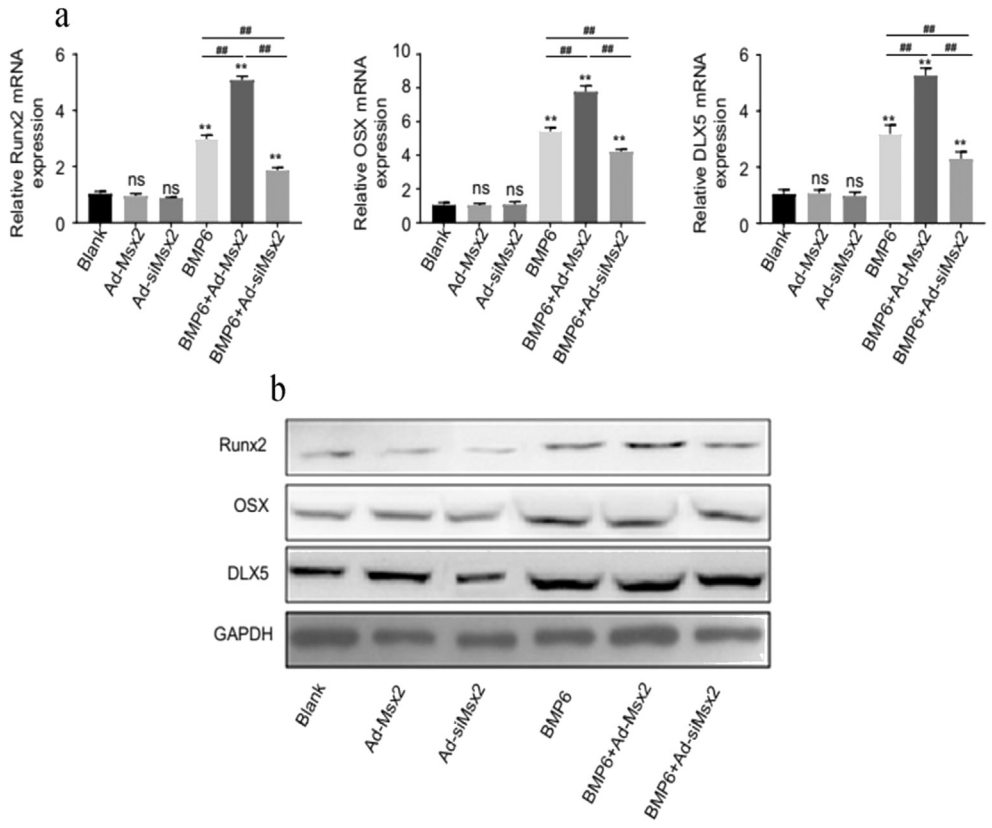

Fig. 3. The mRNA and protein expression of osteogenic markers. The expression levels of Run2, Osx and Dlx5 in each group (A). Protein bands of Runx2, Osx and Dlx5 (B). # indicates $p < 0.05$ between two groups.

that the adenovirus transfection is successful. The transfected-GFP would not affect the expression of Msx2 in MCs.

As an ectoenzyme crucially required for active biomineralization, ALP has been commonly used to evaluate the osteogenic differentiation of stem cells at early stage [34,35]. It was reported that the overexpression of Msx2 could promote the secretion of ALP in MCs [36]. In this work, we observed the similar result with that in previous study. However, the difference between Ad-Msx2 group and blank group was not significant. Notably, BMP6 groups displayed the significantly higher relative activity of ALP than those in Ad-Msx2 groups. Moreover, the relative activities of ALP in BMP6+Ad-Msx2 groups were significantly higher than those in BMP6 groups. This was indicated that high expression of Msx2 could indeed promote BMP6-induced osteogenic differentiation of MCs. Interestingly, the BMP6+Ad-siMsx2 groups also showed the higher relative activity of ALP, compared with blank, Ad-Msx2, and Ad-siMsx2 groups. It implied that in addition to Msx2, BMP6-induced osteogenesis may also involve other mediator, such as Msx1 [37–40].

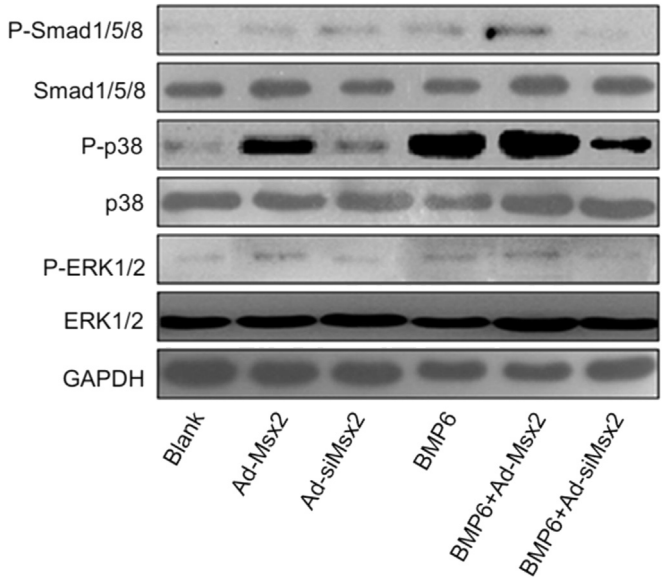

Fig. 4. Western blot of unphosphorylated and phosphorylated proteins in osteogenesis-related signaling pathway.

OPN and OC are the well-known biomarkers of osteogenic differentiation and influence the bone morphology and mechanics [41–43]. The Western blot assay of these two non-collagenous proteins also demonstrated that the overexpressed Msx2 had a potential for facilitating the BMP6-induced osteogenesis. Calcium deposit is regarded as the marker of late osteogenesis [44]. In the present study, the calcium deposits in each group were detected to evaluate the promotion effect of overexpressed Msx2 on BMP6-induced osteogenesis at a late stage. As described in previous studies, the enhanced BMP expression and BMP supplementation could effectively promote the calcium deposit [45,46] which was also observed in our study. The staining of calcium deposits in each group exhibited the similar trend with the staining of ALP, OPN and OC. This meant the facilitation of Msx2 on BMP6-induced osteogenesis also existed at the late stage of osteogenic differentiation.

Furthermore, due to the important role in osteogenic differentiation of stem cells [47,48], the mRNA and protein expression of three bone-related markers including Runx2, Osx and Dlx5 were

also detected in this study. The BMP6-induced osteogenesis of MCs has been proved to require Osx [49]. Moreover, the BMP-induced expression of Osx is mediated by Msx2 [28]. The Dlx and Msx can form heterodimers to regulate the promoter of OC and control the development of limb [50,51]. In the Msx2 deficiency mice model, no expression of Dlx5 was detected [52]. In most occasions, Runx2 is regarded as a partner of Msx2 in regulation of osteogenesis [53]. In this study, the results of both genes and proteins also proved that the BMP6-induced osteogenesis can be strengthened by Msx2.

For gaining further understanding of the mechanism that Msx2 would enhance the BMP6-induced osteogenesis, the MAPK and Smad signaling were detected in the present study because these two signaling pathways were known to involved in osteogenesis [54,55]. In p38 deletion mouse, the osteoblast differentiation is deficient and bone mass is reduced. It also mediates the phosphorylation of Runx2 that is an important event for the osteoblastic differentiation of MCs [56]. In BMP9-induced mineralization, the activity of ALP and phosphorylation of Smad 1/5/8 are abolished by the p38 inhibitor [57]. In addition to the p38 MAPKs, the phosphorylated ERK MAPK signaling also stimulates the osteoblast differentiation [58]. Previous study has demonstrated that ERK MAPK signaling pathway is phosphorylated during the fucoidan-induced osteogenesis [59].

We also found that BMP6+Ad-Msx2 markedly facilitated the phosphorylation of Smad 1/5/8. Similar with the p38 and ERK signaling pathway, Smad 1/5/8 also involve in osteogenic differentiation. Biomechanical stimulation could activate Smad 1/5/8 and furtherly enhance the osteogenesis of osteoblast [60]. The BMP2-induced osteogenic differentiation of C2C12 could be attenuated by strontium via formation of strontium-BMP2 complex and inhibition of Smad-dependent signaling pathway [61]. In the present study, we observed that after transfecting with Msx2 and inducing with BMP6, the phosphorylated Smad 1/5/8, p38 and ERK 1/2 in C3H10T1/2 were the most up-regulated among 6 groups. These results were keeping with those in ALP activity, expression of OPN and OC, calcium deposits, and expression of bone-related genes. Based on the present study, Msx2 is shown to have a potential for enhancing the BMP6-induced osteogenesis. Furthermore, the possible mechanism of this enhancement is that Msx2 facilitates the activation of MAPK and Smad signaling pathways in BMP6-induced MCs.

5. Conclusion

In summary, this study has made a point that the osteogenesis induced by BMP6 could be reinforced with overexpression of Msx2. This reinforced osteogenesis may be mediated by Smad 1/5/8, p38 and ERK 1/2 signaling pathways. However, details in this process are still unclear. All molecules involved in signal transduction should be elucidated as far as possible. If the results obtained from the two mesenchymal cell lines can be applied to the study of tissue-derived mesenchymal stem cells? Therefore, the RNA-seq and Proteomic analyses of each group will be performed in the next step. Furthermore, the following works will focus on the performance of tissue-derived mesenchymal stem cell after various treatments.

Declaration of competing interest

There are no competing interests.

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