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LncRNA SNHG1 modulates p38 MAPK pathway through Nedd4 and thus inhibits osteogenic differentiation of bone marrow mesenchymal stem cells

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ABSTRACT

Aims: Current study aimed to investigate the effects of lncRNA SNHG1 on osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) and explore the underlying mechanisms.

Main methods: Mouse model of osteoporosis was created by ovariectomy (OVX). The BMD of mice spine, the serum level of β-CTX and the ALP activity were measured. The expression of SNHG1, JNK, p-JNK, p-38, p-p38 and Osterix were examined by qRT-PCR and Western blot. Co-IP assay was used to verify the effect of SNHG1 on the interaction between p-p38 and Nedd4. Ubiquitination assay was used to evaluate the roles of SNHG1 in ubiquitination of p-p38.

Key findings: In the mice with osteoporosis, BMD was decreased and β-CTX concentration and SNHG1 expression were increased. ALP activity and p-p38 protein level were elevated and SNHG1 expression was down-regulated in BMSCs stimulated by osteogenic inducer, while the effects were reversed by SNHG1 over-expression. SNHG1 over-expression enhanced the interaction between Nedd4 and p-p38, disrupted protein stability of p-p38, and promoted the ubiquitination of p-p38. In addition, pcDNA-SNHG1 down-regulated p-p38 protein level, and sh-Nedd4 removed the trend. Nedd4 silencing elevated ALP activity and Osterix protein level, while p-38 inhibitor abrogated the effects. In vivo, SNHG1 silence increased BMD and Osterix protein level, and decreased endogenous SNHG1 expression in mice with OVX.

Significance: This study proved the regulation mechanism that lncRNA SNHG1 negatively regulates p38 MAPK signal pathway through ubiquitination mediated by Nedd4, and thus inhibits osteogenic differentiation of BMSCs.

1. Introduction

Osteoporosis is a serious chronic systemic bone disease, which is characterized by low bone mineral density (BMD) and structural disorders [1,2]. There were millions people suffered from osteoporosis, especially postmenopausal women [3]. For the past few years, study has indicated that the abnormal differentiation of bone mesenchymal stem cells (BMSCs) is the most fundamental reason for the occurrence of osteoporosis in postmenopausal women [4]. BMSCs were isolated from the bone marrow and were able to differentiate into osteoblasts, chondrocytes and adipocytes [5]. Previous studies have indicated that the BMSCs could be a suitable cell type in bone repair and remodeling because of its ability of osteogenic differentiation [6]. Hence, how to enhance the osteogenic differentiation of BMSCs has been a hot topic in recent years.

LncRNA has been considered as a novel regulator to control gene expression at transcriptional level, alternative splicing, post- translation regulation and other multiple levels [7]. Up to now, it has been demonstrated that many lncRNAs played crucial roles in osteogenic differentiation, such as lncRNAs MEG3, ANCR and DANCR [8–10]. LncRNA small nucleolar RNA host gene 1 (SNHG1) located on 11q12.3 and was up-regulated in many cancers [11]. In addition, study also revealed that SNHG1 could inhibit proliferation, migration and invasion of osteosarcoma cells by regulating miR-101-3p/ROCK1 pathway [12]. However, whether lncRNA SNHG1 can affect osteogenic differentiation of BMSCs remains inadequately understood.

As a member of MAPK pathway, p38 is an essential regulation factor in regulating various processes, such as inflammation, cell differentiation, cell growth and death [13]. To date, studies have confirmed that the activation of p38 MAPK signal pathway was the crucial trigger factor of osteogenic differentiation of BMSCs [4,14–16]. Previous study also revealed that lncRNAs could inhibit the osteogenic differentiation of BMSCs by suppressing the activation of p38 [17]. Nevertheless, it is still unclear that whether SNHG can disturb the osteogenic differentiation of BMSCs by interfering p38 activation.

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Ubiquitination is an important post-translational regulatory mechanism and mediated by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-ligase (E3). It has been confirmed that the members of E3 family exerts vital functions in osteogenic differentiation, such as WWP2 and Fbw7 [18,19]. As a member of E3 ubiquitin ligases, Nedd4 could suppress the activation of p38 by mediating the polyubiquitination and degradation in osteosarcoma cells [12]. Recent studies also reported that lncRNAs could take part in various diseases through influencing ubiquitination of target proteins [20,21]. Hence, SNHG1 might regulate osteogenic differentiation of BMSCs by Nedd4/p38 MAPK pathway.

Based on those evidences, we speculated that lncRNA SNHG1 might affect osteogenic differentiation via regulating ubiquitination of p38 mediated by Nedd4. In the present study, we aimed to investigate the effects of SNHG1 on osteogenic differentiation and explore the underlying mechanisms.

2. Methods and materials

2.1. Animals

Female mice (10 weeks in age, 18.5–23.2 g in weight) were purchased from Animal Experimental Center, Guangdong Academy of Medical Science (Guangdong, China). All mice were raised in standard pathogen-free conditions: 12 h light/dark illumination cycle, temperature of 20–25 °C and 60% humidity in atmosphere. Mice were kept with free access to food and water. All of them were administrated to acclimatize for a week before starting experiment. Animal experimental procedures were performed in accordance with the Ethics Committee of Department of orthopedics, Qilu Hospital of Shandong University.

2.2. Establishment of animal model

The animal model of osteoporosis was established as previously reported [22]. In brief, mice were divided into ovariectomy (OVX) and sham control groups. Mice were anesthetized with ketamine (40 mg/kg) and rompun (10 mg/kg), and then were fixed and sterilized. Mice skin and muscle were incised on both sides of the dorsal spinal midline. Ovaries on both sides and the surrounding adipose tissue of OVX mice were removed, while only the adipose tissue surrounding ovaries was excised in mice of sham group. After then, the muscle and skin were sutured. 8 weeks after operation, 9 sham mice and 9 OVX mice were sacrificed. Blood samples were collected and bone marrow tissues were separated. All of samples were frozen in liquid nitrogen immediately and stored at −70 °C for further experiments. Other 18 OVX mice were injected with Lenti-NC (n = 9) and Lenti-siRNA-SNHG1 (n = 9) by tail vein. After continuous injection for 3 weeks, mice were euthanized to detect the BMD of spine and expression of SNHG1, Nedd4 and p-p38 in bone marrow tissues.

2.3. Cell culture and induction of osteogenic differentiation

Bone mesenchymal stem cells (BMSCs) were obtained from the BeNa Culture Collection (BNCC, China) and cultured in Alpha Medium with 10% fetal bovine serum, 2 mmol/L L-Glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. BMSCs were stimulated by dexamethasone (Dex, 10^{-8} mol/L), ascorbic acid 2-phosphate (AsAP, 50 μg/mL) and Glycerol 2-phosphate (Gly, 10 mmol/L) to induce steogenic differentiation [23].

2.4. Bone mineral density (BMD) assessment

The mouse spine was collected and fixed with paraformaldehyde for 48 h. Then BMD was assessed by Micro-CT scanning using Latheta LCT-200 [24]. The VGStudio MAX V2.2 3D reconstruction software was used to analyze the data measured.

2.5. ELISA assay of β-CTX

The concentration of serum β-CTX level was examined by using ELISA kits (Phicon, China) according to the manufacturer's instructions.

2.6. Alkaline phosphatase (ALP) assay

ALP activity was measured on the 10th day of induction. BMSCs were washed with Dulbecco's Phosphate Buffer Saline (DPBS) and fixed with 10% Neutral Buffered Formalin for 1 h. Then cells were incubated into BCIP/NBT liquid substrate in dark at room temperature for 10 min. Further, cells were washed, lysed and incubated with buffered substrate at 37 °C for 1 h. 3 N NaOH was added to stop the reactions and ALP activity was evaluated by measuring p-nitrophenol absorbance at 405 nm with a microplate reader (Biorad, USA).

2.7. Cell transfection

BMSCs were seeded in 96-well plates and transfected with SNHG1 over-expression vector (pcDNA-SNHG1), Nedd4 siRNA (sh-Nedd4) and their negative controls using Lipofectamine 2000 (Invitrogen, USA).

2.8. Real-Time Quantitative-PCR

Total RNA was extracted from BMSCs and mouse bone marrow tissues using TRIzol reagent (Invitrogen, USA). RNA was treated with RNase-free DNase I (Invitrogen, USA) to wipe off DNA. The concentration of RNA was detected by a spectrophotometer, and the quality of RNA was determined by agarose gels electrophoresis. RNA (2000 ng) was used to synthesize cDNA using PrimeScript RT reagent kit (Takara, Japan). Real-Time Quantitative-PCR (qRT-PCR) was performed with an ABI Prism 5700 Sequence Detection System (Applied Biosystems) by using UltraSYBR Mixture (CWBIO, China). The relative expression of target genes was calculated by $2^{-\Delta\Delta CT}$ method.

2.9. Western blot

Mouse bone marrow tissues or BMSCs were lysed by RIPA lysis buffer (CWBIO, China) on ice to extract total protein. Concentration of protein was determined by using BCA kits (CWBIO, China). Protein (20 μg) was separated by 10% SDS-PAGE and transferred onto PVDF membrane (Millipore, USA). After then, proteins were incubated overnight with primary antibodies: anti-β-actin (Abcam, 1:1000), anti-Osterix (Abcam, 1:500), anti-p-p38 (Boyao, 1:1000), anti-p-38 (Abcam, 1:1000), anti-JNK (Abcam, 1:1000) and anti-p-JNK (Biocompare, 1:1000) at 4 °C. Further, proteins were incubated with secondary antibodies at room temperature for 1 h. The immunoreactive bands were visualized using the enhanced chemiluminescence reagents (Advansta, USA).

2.10. Co-immunoprecipitation (Co-IP) assay

Anti-bodies of Nedd4 and p-p-38 were used for coimmunoprecipitation and anti-rabbit IgG antibody was considered as a negative control. Protein were extracted with RIPA lysis buffer as described above and incubated with anti-p-p38 or anti-Nedd4 at 4 °C for 24 h, and then added Protein A/G magnetic beads to capture the complex of p-p38/Nedd4. Later, the complex was collected to implement western blot. Finally, immunoreactive bands were visualized using the enhanced chemiluminescence reagents (Advansta, USA).

2.11. Ubiquitination assay

BMSCs were co-transfected with p-p38, pcDNA-SNHG1 and HA-Ubi. 12 h after transfection, cells were treated with proteasome inhibitor MG132 (10 μM) for 12 h. Then cells were lysed by RIPA lysis buffer and

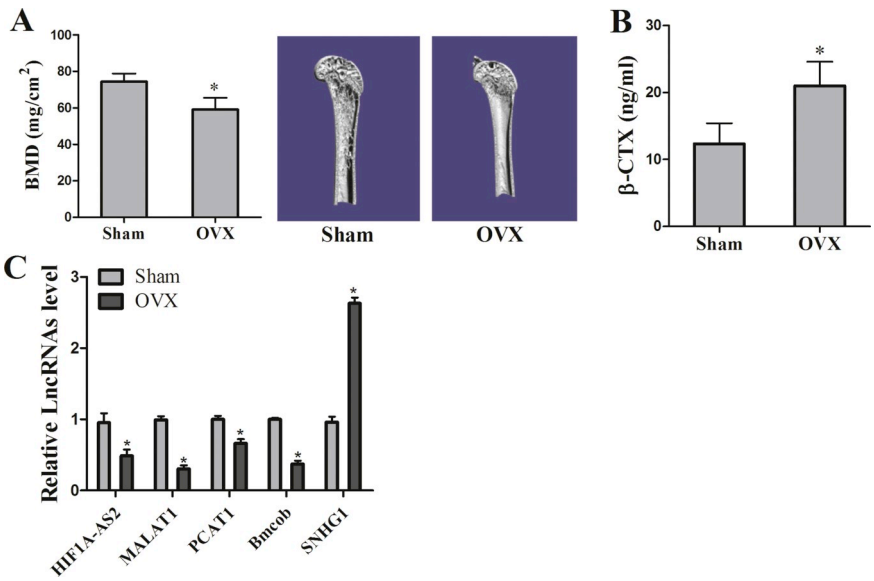

Fig. 1. SNHG1 expression was increased in OVX mice. The osteoporosis model of mouse was established by OVX operation. Mice were divided into sham control (n = 9) and OVX groups (n = 9). (A) BMD of mice spine was assessed by Micro-CT scanning 8 weeks after OVX operation. (B) The serum level of bone conversion marker β-CTX. (C) Expression of lncRNA HIF1A-AS2, lncRNA MALAT1, lncRNA PCAT1, lncRNA Bmcob and lncRNA SNHG1. *P < 0.05 vs. sham group.

boiled for 5 min. Cells lysate was immunoprecipitated with flag antibodies at 4 °C for 24 h and then pulled down with protein A/G beads. Protein samples were separated by SDS-PAGE and analyzed by immunoblotting as described above.

2.12. Statistical analysis

Data was analyzed using SPSS 20.0 and expressed as mean ± SD. Student's t-test was used to compare the difference between two groups. P < 0.05 indicated significant differences of data.

3. Results

3.1. SNHG1 expression were increased in OVX mice

Compared with sham mice, Micro-CT scanning results showed marked decrease of BMD in OVX mice (Fig. 1A, P < 0.05). The serum level of bone conversion marker β-CTX was evidently elevated in OVX mice (Fig. 1B, P < 0.05). Additionally, we also detected expression of lncRNAs which played crucial roles in osteogenic differentiation. The relative levels of HIF1A-AS2, MALAT1, PCAT1 and Bmcob were significantly decreased in OVX mice, while the expression of SNHG1 was remarkably increased in OVX mice (Fig. 1C, P < 0.05).

3.2. SNHG1 expression was down-regulated in osteogenic inducer-stimulated BMSCs

BMSCs were divided into control and induced group. BMSCs were treated with DEX, AsAP and Gly to stimulate osteogenic differentiation. On the 10th day of induction, ALP activity was notably enhanced in induced group compared with control (Fig. 2A, P < 0.05). However, the induction markedly reduced the expression of SNHG1 (Fig. 2B, P < 0.05).

3.3. SNHG1 attenuated p38 activation and osteogenic differentiation

To test the effects of SNHG1 on osteogenic differentiation, BMSCs were transfected with pcDNA-SNHG1 or pcDNA and stimulated by osteogenic inducer. Then the expression of SNHG1, p38, p-p38, JNK, p-JNK and Osteoblast-specific transcription factor (Osterix) were examined and ALP activity was measured. For SNHG1, osteogenic inducer significantly depressed its expression, while pcDNA-SNHG1 removed the effect (Fig. 3A, P < 0.05). Compared with control, protein level of p-p38 and p-JNK were increased in induced group, while pcDNA-

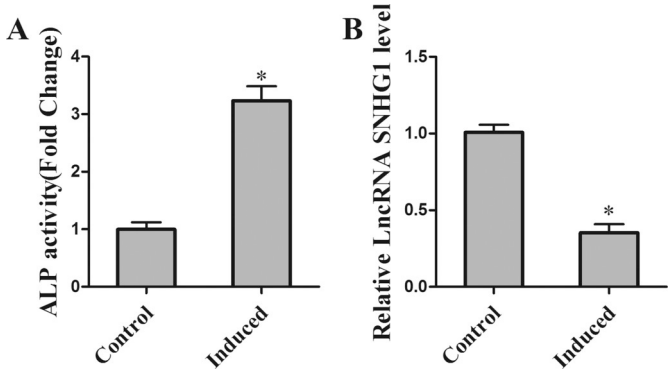

Fig. 2. SNHG1 expression was down-regulated in BMSCs stimulated by osteogenic inducer. BMSCs were treated with DEX (10^{-8} mol/L), AsAP (50 μg/mL) and Gly (10 m mol/L) and divided into 2 control and Induced groups. (A) ALP activity in BMSCs was measured on the 10th day of induction. (B) Relative expression of lncRNA SNHG1 detected by qRT-PCR. *P < 0.05 vs. control group.

SNHG1 down-regulated p-p38 protein level only (Fig. 3B). Besides, ALP activity was notably facilitated by osteogenic inducer, and pcDNA-SNHG1 reversed the trend (Fig. 3C, P < 0.05). As well, the protein level of Osterix was raised by osteogenic inducer, and pcDNA-SNHG1 eliminated the effect (Fig. 3D).

3.4. SNHG1 reduced p-p38 protein level through Nedd4

BMSCs were transfected with pcDNA-SNHG1 or pcDNA and treated with osteogenic inducer. Co-IP assay was performed to investigate the interaction between SNHG1, p-p38 and Nedd4. Results of Co-IP assay showed that Nedd4 recruited more p-p38 protein in BMSCs transfected with pcDNA-SNHG1 than pcDNA. Meanwhile, SNHG1 over-expression also boosted the recruitment of p-p38 to Nedd4 protein (Fig. 4A, P < 0.05).

To evaluate the effect of SNHG1 on p-p38 protein stability, BMSCs were transfected with pcDNA-SNHG1 or pcDNA, and treated with osteogenic inducer and protein synthesis inhibitor (CHX, 50 μg/mL). Compared with pcDNA, SNHG1 over-expression significantly reduced the protein stability of p-p38 after CHX treatment (Fig. 4B, P < 0.05).

BMSCs were transfected with pcDNA-SNHG1, sh-Nedd4 and their own negative control, and treated with osteogenic inducer to verify whether SNHG1 targets p-p38 through Nedd4. As shown in Fig. 4C, SNHG1 over-expression reduced p-p38 protein level, while Nedd4

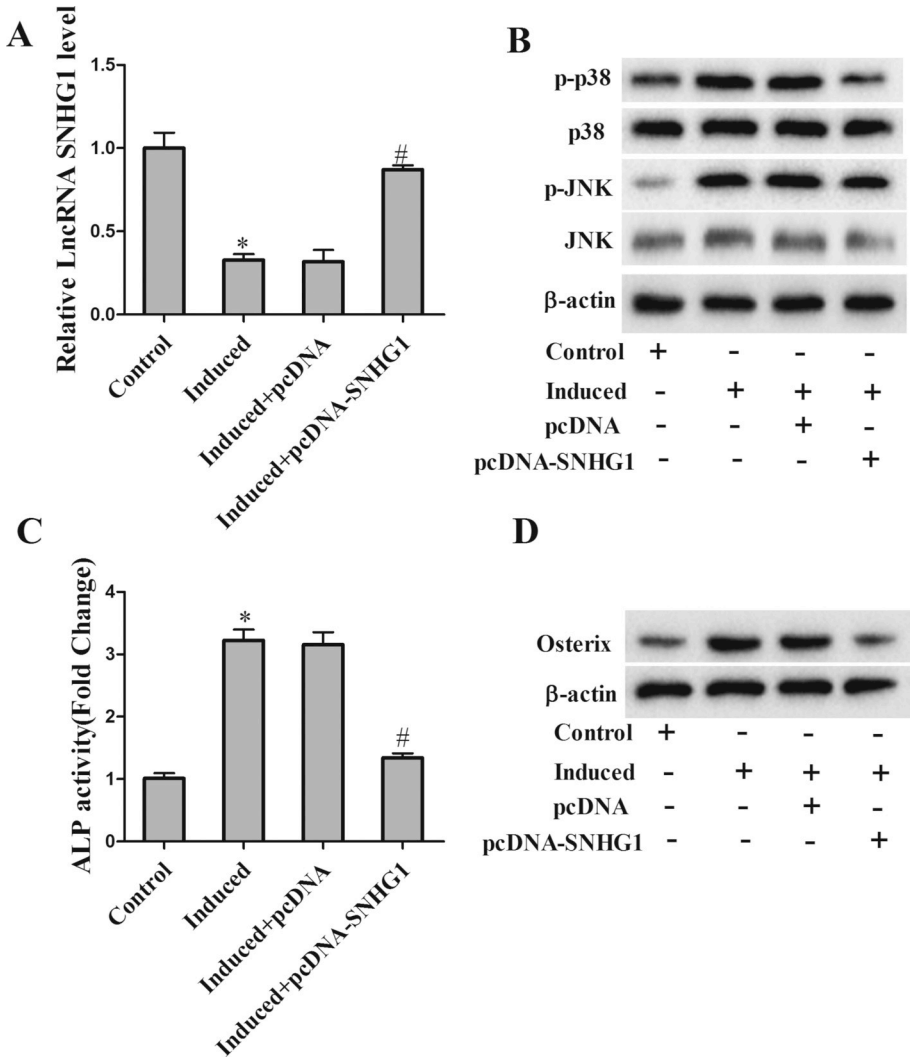

Fig. 3. SNHG1 attenuated p38 activation and osteogenic differentiation. BMSCs were transfected with pcDNA-SNHG1 or pcDNA and stimulated by osteogenic inducer. Cells were divided into 4 groups: control, Induced, Induced + pcDNA and Induced + pcDNA-SNHG1. (A) Relative expression of lncRNA SNHG1 detected by qRT-PCR. (B) Protein level of p-p38, p38, p-JNK and JNK determined by western blot. (C) ALP activity in BMSCs. (D) Protein level of Osterix determined by western blot. $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. induced + pcDNA group.

silencing abrogated the trend.

Further, we assessed the effect of SNHG1 on ubiquitination of p-p38. The results showed that SNHG1 facilitated the ubiquitination and reduced the protein level of p-p38 (Fig. 4D).

3.5. SNHG1 inhibited osteogenic differentiation by modulating Nedd4/p38 MAPK pathway

In order to elucidate the effects of p38 on osteogenic differentiation, BMSCs were transfected with pcDNA-SNHG1 sh-Nedd4 and sh-control and treated with p-38 MAPK inhibitor (SB203580, 10 μM). Then the ALP activity and the proein level of Osterix were detected. The results showed that silence of Nedd4 significantly increased ALP activity, while p-38 MAPK inhibitor reversed the effect (Fig. 5A, $P < 0.05$). In addition, sh-Nedd4 transfection up-regulated the protein level of Osterix, and SB203580 removed the trend (Fig. 5B).

3.6. Silence of SNHG1 alleviated osteoporosis by promoting p-38 activation in vivo

OVX mice were continuously injected with Lenti-NC or Lenti-siRNA-SNHG1 for 3 weeks to verify the effects of SNHG1 on osteogenic differentiation in vivo. We found that Lenti-siRNA-SNHG1 injection significantly augmented the BMD of OVX mice compared with Lenti-NC group (Fig. 6A). As well, the expression of endogenous SNHG1 and the protein level of Nedd4 were down-regulated by Lenti-siRNA-SNHG1 injection (Fig. 6B). Besides, Lenti-siRNA-SNHG1 caused an increase of p-p38 protein level and a decrease of Nedd4 protein level (Fig. 6C).

4. Discussions

Osteoporosis is widely known as a serious and complicated bone disease with high risk of fracture [1]. As shown in the previous and present studies, patients with osteoporosis have a low BMD and high β-CTX level [24]. Recent years, it was been proved that osteoporosis in postmenopausal women mainly caused by the abnormal osteogenic differentiation of BMSCs [4]. Thus, enhancing the osteogenic differentiation of BMSCs plays important roles in preventing the occurrence of osteoporosis.

Recently, documents reported that lncRNAs played crucial roles in osteogenic differentiation [9,10,12]. Studies have testified that lncRNAs HIF1A and MALAT1 could promote the osteogenic differentiation of BMSCs [25,26]. In the present study, we examined the relative level of five lncRNAs in mouse model of osteoporosis. Results

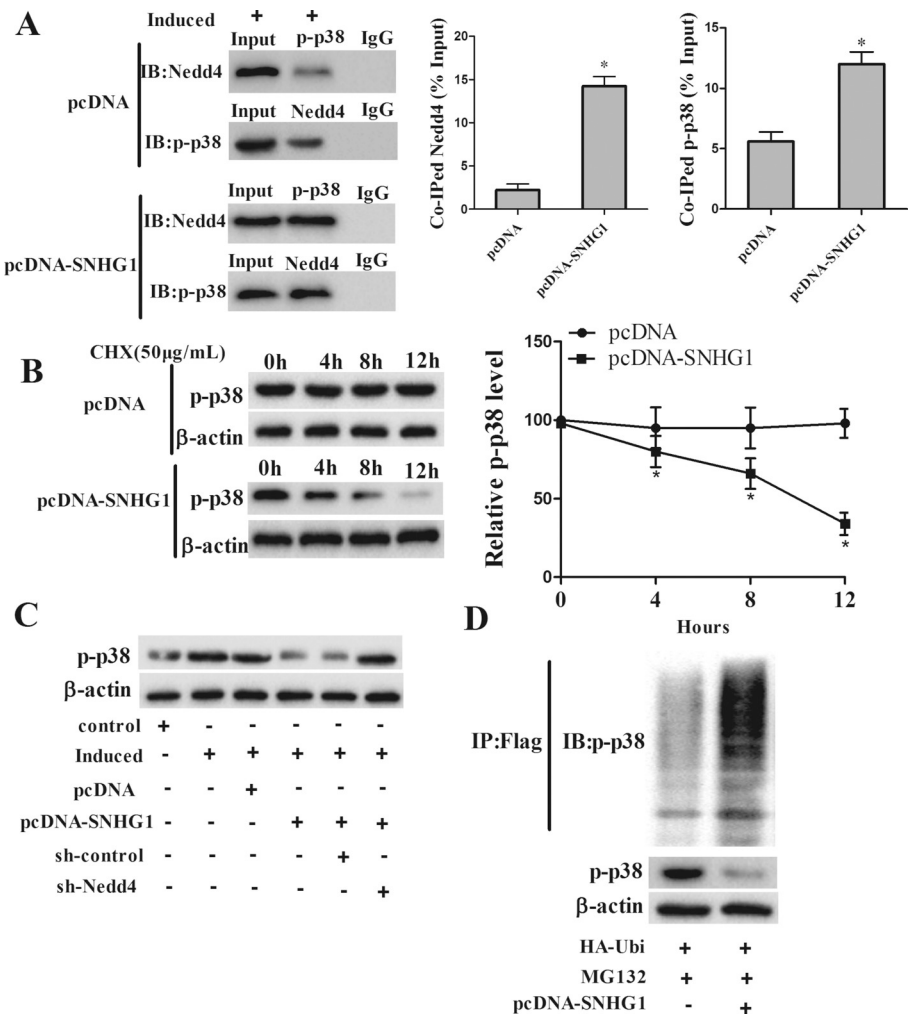

Fig. 4. SNHG1 reduced p-p38 protein level through Nedd4. (A) BMSCs were transfected with pcDNA-SNHG1 or pcDNA and treated with osteogenic inducer. Co-IP assay was performed with antibodies of p-p38 and Nedd4. Then the expression of p-p38 and Nedd4 were examined. (B) BMSCs were transfected with pcDNA-SNHG1 or pcDNA and treated with osteogenic inducer and protein synthesis inhibitor (CHX, 50 μg/mL). The protein stability of p-p38 was evaluated. (C) BMSCs were transfected with pcDNA-SNHG1, sh-Nedd4 and their own negative control and treated with osteogenic inducer. The protein level of p-p38 was determined by western blot. (D) Immunoblot analysis of p-p38 ubiquitylation. *P < 0.05 vs. pcDNA group.

showed that the expression of HIF1A-AS2, MALAT1, PCAT1 and Bmcob were lower in OVX mice than in sham mice, while the expression of SNHG1 was much higher in OVX mice than in sham mice. That might suggested the different effects of SNHG1 on osteogenic differentiation compared with other four lncRNAs. We also observed that osteogenic inducer inhibited the expression of endogenous SNHG1 in BMSCs and improved the expression of Osterix and ALP activity, while the overexpression of SNHG1 reversed the effects. These results convinced us that SNHG1 could inhibit osteogenic differentiation.

MAPK signal pathways, including JNK, ERK 1/2 and p38, exert significant functions in osteogenic differentiation. Normally, MAPK p38, JNK and ERK 1/2 are activated to trigger osteogenic differentiation, while the inactivation of MAPK can result in the imbalance of osteogenic differentiation [15,16]. The protein level of p-p38 and p-

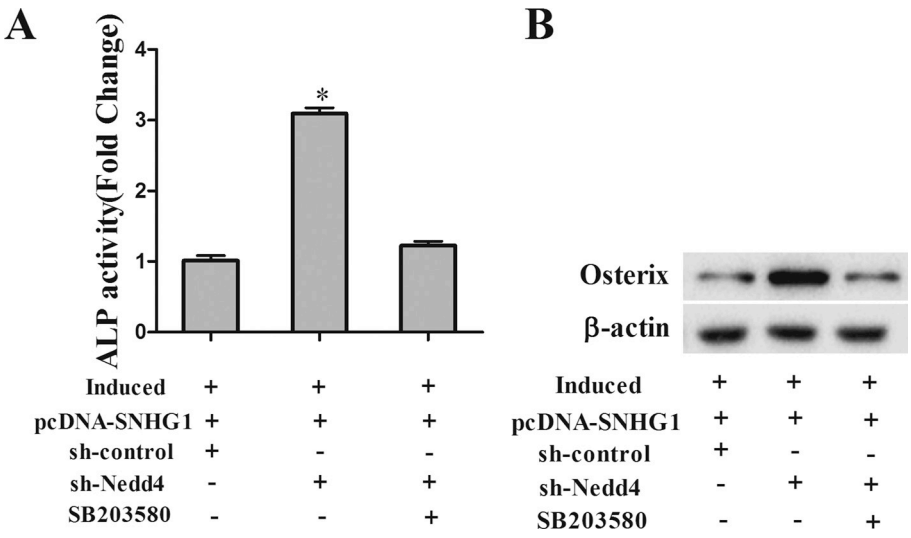

Fig. 5. SNHG1 inhibited osteogenic differentiation by modulating Nedd4/p38 MAPK pathway. BMSCs were transfected with pcDNA-SNHG1, sh-Nedd4 and sh-control and treated with p-38 MAPK inhibitor (SB203580, 10 μM). BMSCs were divided into 3 groups: Induced + pcDNA-SNHG1 + sh-control, Induced + pcDNA-SNHG1 + sh-Nedd4 and Induced + pcDNA-SNHG1 + sh-Nedd4 + SB203580. (A) ALP activity in BMSCs. (B) Osterix protein level in BMSCs detected by western blot. *P < 0.05 vs. Induced + pcDNA-SNHG1 + sh-control group.

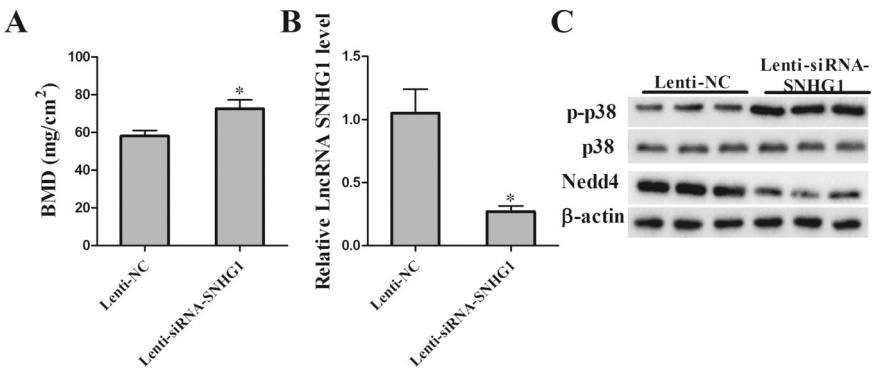

Fig. 6. Silence of SNHG1 alleviated osteoporosis by promoting p-38 activation in vivo. The osteoporosis model of mice was established by OVX, and OVX mice were continuously injected with Lenti-NC and Lenti-siRNA-SNHG1 for 3 weeks (n = 9). (A) BMD of mice spine was assessed by Micro-CT scanning. (B) Relative expression of lncRNA SNHG1 in bone marrow tissues. (C) Protein level of p-p38, Nedd4 and p-38 in bone marrow tissues detected by western blot. *P < 0.05 vs. Lenti-NC group.

JNK were both increased in BMSCs by the stimulation of osteogenic inducer in this study. That might imply the positive effect of p38 and JNK activation on osteogenic differentiation. Additionally, previous studies also mentioned that lots of lncRNAs were closely related to p38 MAPK pathway [27,28]. Most importantly, the activation of p38 stimulated by silence of lncRNA DANCR induced differentiation of MSCs into osteoblasts [12]. In current research, the increase of p-p38 protein level, but not p-JNK protein level, was abrogated by pcDNA-SNHG1 transfection. That revealed that SNHG1 regulated osteogenic differentiation of BMSCs by suppress the activation of p38 rather than JNK. Moreover, injection of Lenti-siRNA-SNHG1 increased the BMD of spine and the protein level of p-p38, and repressed the expression of endogenous SNHG1 in OVX mice. These results further supported the point that SNHG1 affected osteogenic differentiation by modulating p38 MAPK pathway.

As a vital post-translational regulatory mechanism, ubiquitination of proteins exhibits crucial roles osteogenic differentiation and other biological functions. In general, ubiquitination is mediated by three classes of key enzymes: E1, E2 and E3. Nedd4 is a member of E3 family, and participates in ubiquitination progresses of diversified proteins [29,30]. Furthermore, Nedd4 depressed the expression of p38 by inducing the polyubiquitination and degradation [8]. It was also verified that lncRNAs could affect target molecules via ubiquitination. In this study, the Co-IP assay showed SNHG1 over-expression enhanced the interactions between p-p38 and Nedd4. As well, SNHG1 over-expression declined the stability of p-p38 in BMSCs. That demonstrated that SNHG1 might promote the combination of p-p38 to Nedd4 and accelerate the degradation of p-p38. Subsequently, sh-Nedd4 transfection eliminated the inhibitor effect of pcDNA-SNHG1 on p-p38 expression. The ubiquitination assay exhibited that SNHG1 over-expression facilitated the ubiquitination and reduced the protein level of p-p38. Those findings manifested that SNHG1 regulated p38 pathway via ubiquitination mediated by Nedd4. Further, sh-Nedd4 transfection augmented ALP activity and Osterix expression, while p38 specific inhibitor SB203580 abolished the trends. Hence, those results were more convincing and direct evidence to suggest the idea above.

Taken together, lncRNA SNHG1 could suppress p38 activation through ubiquitination mediated by Nedd4, and thus inhibited osteogenic differentiation of BMSCs. The current study also suggested that inhibiting lncRNA SNHG1 might be a potential therapeutic target to enhance osteogenic differentiation of BMSCs.

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Conflict of interest

All authors declare that they have no conflicts of interest to this work.

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