Lysyl Oxidase Down-Regulates The Osteoblastic Potential of BMP9 Through Inhibiting HIF-1α/Wnt/β-Catenin Axis in Mesenchymal Stem Cells

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Research

Keywords: Lysyl oxidase, BMP9, Osteogenic differentiation, HIF-1α, Wnt/β-catenin

Posted Date: May 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-509252/v1

Abstract

Background: Bone morphogenetic protein 9 (BMP9) is one of excellent osteogenic factors, but it can also initiate adipogenesis concomitantly. Thus, the osteogenic potential of BMP9 may be enhanced if the adipogenesis were reduced. It was reported that lysyl oxidase (Lox) may function as a critical switcher for adipogenesis. Up to date, the role of Lox in BMP9-induced osteoblastic differentiation remains unknown.

Methods: The effect and possible mechanism of Lox on the osteogenic function of BMP9 were evaluated with RT-PCR, western blotting, immunofluorescent and histochemical staining. The same results were also confirmed with the in vivo BMP9-induced ectopic bone formation model.

Results: The mRNA and protein of Lox are both detectable in progenitor cells, and it was increased by BMP9 in 3T3-L1 cells. BMP9-induced Runx2, OPN and mineralization were all enhanced by inhibiting or silencing Lox, but reduced by exogenous Lox. BMP9 increased the mRNA level of c-Myc, which was enhanced by inhibiting Lox, so did the protein level of β-catenin. Effects of Lox specific inhibitor on BMP9-induced Runx2, OPN and mineralization were reduced obviously by silencing β-catenin. HIF-1α was up-regulated by BMP9, which was enhanced by inhibiting or silencing Lox, but decreased by Lox over-expression. The effects of Lox specific inhibitor on increasing BMP9-induced osteogenic markers were reduced greatly by silencing HIF-1α. On the contrary, the inhibitory effect of Lox on BMP9-induced osteoblastic markers was almost abolished by HIF-1α over-expression. BMP9-induced bone formation was increased by silencing Lox or over-expressing HIF-1α. The effect of silencing Lox on potentiating BMP9-induced bone formation was attenuated by silencing HIF-1α. Lox specific inhibitor increased the level of β-catenin and decreased that of SOST, but these effects were almost reversed by silencing HIF-1α.

Conclusions: Lox may reducing the osteoblastic-induction function of BMP9 through inhibiting Wnt/β-catenin signal via down-regulation of HIF-1α partly.

1. Introduction

Multipotent progenitor cells can be committed into various lineages, such as osteoblastic, condrogenic, adipogenic, or myogenic, dependent on the context or specific induction from special cytokines(1). For osteogenesis is related with adipogenesis closely, the promotion of osteogenesis usually based on the cost of adipogenesis, and vice verse. Bone morphogenetic proteins (BMPs) is a serial of factors belong to TGF-β super-family, and several member of BMP possess excellent potential to commit progenitor cells to osteoblastic lineage, such as BMP2, BMP7 and BMP9(2, 3). To date, expanding evidences support that BMP9 is one of most effective factors to induce osteoblastic commitment, and may be a potent alternative for bone tissue engineering(4, 5). However, adipocytes are also presented in the bone masses induced by BMP9 obviously (6). Thus, the osteogenic ability of BMP9 may be strengthened on condition that the adipogenic process was inhibited.

There are several critical factors which related with promoting adipogenic lineage commitment from progenitor cells, such as peroxisome proliferator activated receptor gamma (PPARγ) and C/EBP-a(7). It

was reported that BMP9 can up-regulate PPARγ during the osteogenic process, but silencing PPARγ could reduced the osteogenic potential of BMP9 substantially. This evidence suggested that PPARγ may also play an important role in the osteogenic process induced by BMP9. All-trans retinoic acid can enhance the osteogenic potential of BMP9, one of the reasons may be resulted from inhibiting adipogenic differentiation, although the exact mechanism keeps unknown (8). Therefore, there may exist some special factors which can inhibit adipogenic differentiation but enhance osteogenic potential of BMP9 simultaneously.

Lysyl oxidases (Lox) is a copper-dependent enzyme. Its function is involved in the metabolism of lysine or lysine residues by converting it to active product, such as aldehydes, and then made cross-link of the extra-cellular protein(9). This process may be very important for the stabilization of collagen fibrils or the elasticity of elastin (10, 11). For this reason, Lox plays an very important role in the development, such as skin and respiratory system(12). The function loss or over-expression of Lox is implicated with many diseases, such as myelofibrosis, lung cancer, and metastasis of cancer cells(13–15). Besides, Lox also play crucial role in regulating proliferation and differentiation. It was reported that Lox may control the formation of adipocyte from progenitor cells(16, 17). Thus, Lox may be a target to enhance the osteoblastic potential of BMP9. However, it remains unknown whether the BMP9-induced osteogenic differentiation is associated with Lox, or the function loss of Lox may promote the osteogenic potential of BMP9 through decreasing adipogenesis.

Herein, we analyzed the effect of BMP9 on Lox in progenitor cells, and determined the effect of Lox on the ostogenic potential of BMP9, as well as the possible molecular mechanism which involved in this physiological process.

2. Materials And Methods

2.1 Cell culture and chemicals

C3H10T1/2, C2C12, 3T3L-1, and HEK-293 cell lines were purchased from ATCC. MEFs was extracted from embryo of NIH mouse. Lox specific inhibitor, β-aminopropionitril (BAPN, #T13475) was purchased from Topscience Co., Ltd (Shanghai, China), and dimethyl sulfoxide was used as solvent. Cells were cultured with complete DMEM medium (containing 10% fetal bovine serum, 100 kU/L penicillin and 0.1 g/L streptomycin) at 37 ℃ with 5% CO_2. Primary antibodies against GAPDH (10494-1-AP) and sclerostin (21933-1-AP) were ordered from proteintech (China branch); primary antibodies against BMP9 (sc-514211), LOX (sc-32409), Runx2 (sc-390351), OPN (sc-21742), β-catenin (sc-7963), H_2A_x (sc-517336) and HIF-1α (sc-13515) were all purchased from Santa Cruz Biotechnology (China branch).

2.2 Construction of recombinant adenovirus vector

Recombinant adenovirus vectors for this study was constructed with AdEasy system(18, 19). Briefly, coding sequence of the target gene was amplified with PCR using high-fidelity Taq-polymerase, and cloned into the special shuttle plasmid. Then, homologous recombination is carried out between the

linearized shuttle plasmid and the adenovirus skeleton plasmid. Finally, the correct plasmid product is linearized and transfected into HEK-293 cells for packaging target recombinant virus.

2.3 Alkaline phosphatase (ALP) activity assay

For ALP assay, cells were plated in 24-well plates, and then treated with corresponding reagents according to experimental design after cells attach well. On day 5 and 7, the NBT/BCIP kit (#C3206, Beyotime, Shanghai, China) was used for measuring the ALP activities. Each experiment was repeated in at least three independent experiments.

2.4 Mineral deposition assay

For this assay, cells were seeded in 24-well plates, and were treated with appropriate reagents according to experimental design. Forty-eight hours after the initial treatment, the medium was replaced with osteogenic-induction medium(20). On day 20, the culture medium was discarded, and the cells were washed gently with phosphate buffered saline (PBS), fixed with 0.05% glutaraldehyde for 10 min, washed with PBS of pH 4.2 gently after discarding the fixing solution. Then, cells were stained for 5 min with Alizarin red S solution (0.4%). Finally, discard the Alizarin red S solution and wash the cells with PBS (pH 4.2) for three times. The plate was scanned and images were taken under a microscopy. Each experiment was repeated in at least three independent experiments.

2.5 Total RNA extraction, reverse transcription (RT) and polymerase chain reaction (PCR) assay

The exponentially growing cells were seeded in the six-well plate and treated with the appropriate reagents according to the experimental design. At the pre-determined time point, total RNA was extracted with TRIzol reagent, and cDNA was produced with RT. Then, the cDNA product was diluted 5 to 10 folds and used as template for mRNA expression level assay with PCR. All results were normalized with the mRNA level of glyceraldehyde phosphate dehydrogenase (GAPDH). The primers used in this experiment are shown in Table 1.

Table 1
The primers used for PCR

Gene	Primer	Sequence (5’ →3’)
Lox	F	TGCCAACACACAGAGGAGAG
	R	CCAGGTAGCTGGGGTTTACA
Runx2	F	GCCAATCCCTAAGTGTGGCT
	R	AACAGAGAGCGAGGGGGTAT
ALP	F	AGACCAGGTCTGCTCAGGAT
	R	ACCCCGCTATTCCAAACAGG
c-Myc	F	GCCCAGTGAGGATATCTGGA
	R	ATCGCAGATGAAGCTCTGGT
HIF-1α	F	CTGGGACTTTCTTTTACCATGC
	R	AATGGATTCTTTGCCTCTGTGT
GAPDH	F	ACCCAGAAGACTGTGGATGG
	R	CACATTGGGGGTAGGAACAC
F: forward; R: reverse		

2.6 Western blot analysis

Exponentially growing cells were seeded in the six-well plate and treated with various factors according to the experimental design, proteins were extracted at the corresponding time point with radio immunoprecipitation assay (RIPA) lysis buffer (#R0020-100 Solarbio, China). Target proteins were separated with SDS-PAGE, transfered to polyvinylidene difluoride membrane, blocked with 5% bovine serum albumin, and probed with the corresponding primary anti-body. Finally, the ECL method is used for development, and a Bio-Rad gel imager is used for imaging.

2.7 Immunofluorescence assay

Cells were seeded in a 48-well plate evenly, and various factors were added to treat the cells according to experimental design after cells attach to the wall. After treating for 48 h, the cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS; treated with 0.5% Triton X-100 for 20 min, washing with PBS; blocked with goat serum at 37 ˚C for 30 min, then incubated with primary antibody at 4 ˚C overnight. Cells were washed with PBS and incubated with fluorescent secondary antibody (#A23410-1, Abbkine) at 37 ˚C for 30 min, were washed three times with PBS, and incubated with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) for 10 min. Finally, images were taken with microscope.

2.8 Ectopic osteogenesis assay

The nude mice were ordered from the Experimental Animal Center of Chongqing Medical University (five per group, 6-week-old, females, and body weight is 20–24 g). This experiment was approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (Chongqing, China). Cells were cultured in 100 mm dishes, and pre-treated with corresponding reagents. After 36 hours, cells were collected, centrifuged and resuspended with PBS (4 ˚C). Finally, cells were implanted to the flanks of mice subcutaneously (5×10^6 cells per injection). Four weeks later, the nude mice were all euthanized and the bone tissues were collected for the following micro-CT scan and histological evaluation.

2.9 Analysis of Micro-computed tomographic (μ-CT)

Sample of bone masses were scanned with micro-CT (SCANCO Medical AG, Switzerland). Then, the data were analyzed with μ-CT 516.1 (provided by the scanner manufacture) for reconstruction of three dimensional and quantification.

2.10 Tissue section preparation, staining and evaluation

The retrieved bone samples were fixed with 4% paraformaldehyde for 48 hours and decalcified. Then, embedded with paraffin for preparing sections. Finally, the sections were subjected with hematoxylin and eosin (H&E) staining.

2.11 Immunoprecipitation assay

Cells in the logarithmic stage were seeded in a six-well plate, and treated with corresponding the reagent according to experimental design. After 36 hours, the culture medium was discarded, and RIPA lysis buffer (300 μl per well) containing protease and phosphatase inhibitor were added to treat the cells on on ice for 30 minutes. The lysate were collected and centrifugated at 4 ˚C for 5min (14000 g). The supernatant were harvested and incubated with pretreated magnetic beads for 60 minutes at 4 ˚C. The supernatant was collected and incubated with corresponding primary antibody overnight, incubated with the pretreated magnetic beads at 4 ˚C for 4 hours. The magnetic beads were collected and washed with RIPA lysis buffer for three times, and boiled with RIPA lysis buffer and β-mercaptoethanol for 10 min. Finally, the samples were subjected to the regular western blot analysis.

2.12 Statistical analysis

The data were analyzed with GraphPad Prism 6 software, and the data were showed as mean ± standard deviation. Student *t*-test was used to analyze the difference between groups. The difference was defined as statistical significance if the *p* value is less than 0.05.

3. Results

3.1 BMP9 affects the expression of lox in multiple progenitor cells

Real-time PCR assay results indicated that the mRNA of Lox is detectable in C3H10T1/2, MEFs, 3T3-L1 and C2C12 cells (Fig. 1A), and the protein of Lox were also measurable in these cell lines (Fig. 1B and C). Although BMP9 is an excellent osteogenic factor, the relationship between BMP9 and Lox remains unknown yet. Real-Time PCR results show that the mRNA level of Lox was increased greatly by BMP9 in 3T3-L1 cells (Fig. 1D), western blot assay showed the similar results (Fig. 1E and F). These evidences suggested that Lox may be associated with the regulation of BMP9's osteogenic capability in multiple progenitor cells.

3.2 Lox affects the osteogenic markers induced by BMP9 in 3T3-L1 cells

Next, we determined whether Lox can affect the osteogenic induction potential of BMP9. Real-time PCR results showed that the BMP9-induced mRNA of Runx2 was increased by Lox specific inhibitor substantially in 3T3-L1 cells (Fig. 2A), the protein level of Runx2 induced by BMP9 was also elevated by this inhibitor (Fig. 2B). ALP activity assay results showed that Lox specific inhibitor enhanced the BMP9's effect on inducing the ALP activities in 3T3-L1 cells (Fig. 2C and D). Similar results were found in the BMP9-induced protein level of OPN and mineralization (Fig. 2E and F). Besides mineralization, the BMP9-induced protein level of Run2 and OPN were increased by silencing Lox in 3T3-L1 cells (Fig. 2G and H). However, over-expression of Lox reduced the potential of BMP9 to induce Runx2, OPN, and mineralization in 3T3-L1 cells (Fig. 2J, K and L). These data indicated that Lox may play as a negatively regulator for the osteoblastic function of BMP9, but the mechanism underlying this process keeps unclear yet.

3.3 Effect of Lox and/or Wnt/β-catenin on the osteoblastic markers induced by BMP9 in 3T3-L1 cells

Real-time PCR results show that BMP9 increases the mRNA level of c-Myc, one downstream target of Wnt/β-catenin signal, Lox specific inhibitor reduced the level of c-Myc; but the mRNA level of Lox was increased greatly by the combination of BMP9 and Lox specific inhibitor (Fig. 3A). Western blot results show the protein level of β-catenin was increased by BMP9, and the Lox specific inhibitor exhibited no substantial effect on it; but the protein level of β-catenin was increased obviously by the combination of BMP9 and Lox specific inhibitor (Fig. 3B). BMP9 increased the protein level of Runx2 and OPN, so did the mineralization; these effect of BMP9 were increased by Lox specific inhibitor, but reduced by the combination of β-catenin knockdown (Fig. 3C, D, and E). These data suggested that the regulatory effect of Lox on the BMP9's osteogenic capability may be achieved by affecting the activity of Wnt/β-catenin signal.

3.4 BMP9 and/or Lox affects the expression of HIF-1α in 3T3-L1 cells

Western blot results show the protein level of HIF-1α was increased by BMP9 markedly in 3T3-L1 cells (Fig. 4A, B, and C). The potential of BMP9 on increasing mRNA and protein level of HIF-1α was also increased by the Lox specific inhibitor (Fig. 4D, E, and F). Immunofluorescent staining results also show the effect of BMP9 on inducing HIF-1α can be elevated by Lox specific inhibitor (Fig. 4G). Similarily, the effect of BMP9 on inducing HIF-1α can be elevated by silencing Lox, but attenuated by over-expressing Lox (Fig. 4H and I). These data suggested that the regulatory effect of Lox on BMP9's osteogenic potential may be associated with HIF-1α.

3.5 HIF-1α affects the effect of Lox on the osteoblastic markers induced by BMP9 in 3T3-L1 cells

Western blot assay results show the BMP9-induced Runx2 was increased by Lox specific inhibitor, but this effect was almost diminished by silencing HIF-1α (Fig. 5A). Similar results were reproduced on the BMP9-induced ALP activity, protein level of OPN, and matrix mineralization (Fig. 5B, C and D). On the contrary, western blot analysis results show the effect of BMP9 on inducing Runx2 was inhibited by exogenous Lox, and this effect was almost rescued by exogenous HIF-1α (Fig. 5E). Similarily, the BMP9-induced ALP activity, protein level of OPN and matrix mineralization were affected by over-expressing Lox and/or HIF-1α with the same way (Fig. 5F, G and H). These data indicated the regulatory effect of Lox on the BMP9's osteoblastic activity was partly realized through HIF-1α.

3.6 Lox and/or HIF-1α affects the bone formation induced BMP9 in 3T3-L1 cells

The 3D re-construction of u-CT scanning data showed that the osteogenic capability of BMP9 was increased by silencing Lox or over-expressing HIF-1α, but the effects of Lox knockdown on enhancing the osteogenic potential of BMP9 was partly inhibited by silencing HIF-1α (Fig. 5A). The quantitative analysis of the u-CT scanning data showed the similar results (Fig. 5B). H&E staining results also showed that Lox knockdown increased the trabecular bone induced by BMP9, which was reduced by silencing HIF-1α apparently (Fig. 5C). These data suggested the effect of Lox on the osteoblastic capability of BMP9 may be partly mediated by HIF-1α.

3.7 Lox and/or HIF-1α affects the effect of BMP9 on Wnt/β-catenin pathway

Further western blot analysis results showed that Lox knockdown increased the protein level of β-catenin in nucleus and reduced its level in cytoplasm (Fig. 7A and C). BMP9 increased the protein level of sclerostin (SOST) in 3T3-L1 cells, which was reduced by Lox specific inhibitor (Fig. 7D and E). However, the inhibitory effect of Lox specific inhibitor on the BMP9-induced SOST was partly reversed by silencing HIF-1α (Fig. 7F and G). Western blot results show that the BMP9-increased protein level of β-catenin was potentiated by Lox specific inhibitor, which was notably reduced by silencing HIF-1α (Fig. 7H and I). These data suggested that the effect of Lox on Wnt/β-catenin signal may be realized through HIF-1α partly.

4. Discussion

In this study, we demonstrated that BMP9 up-regulates Lox in preadipocyte, the osteoblastic capability of BMP9 was strengthened by inhibiting or silencing Lox, but reduced by Lox over-expressing. The possible molecular mechanism underlying this process may be resulted from increasing the activity of Wnt/β-catenin signal through up-regulating HIF-1α when Lox was inhibited or knockdown.

It's well known that multiprogenitor cells can be committed toward different lineages, including adipogenic, osteogenic, myogenic, and chondrogenic lineages. In the development of skeletal system, adipogenesis and osteogenesis may occurred concomitantly although they are excluded usually. The homostasis of bone is affected by the balance between adipogenic and osteoblastic differentiation. More osteogenesis may dependent on the cost of adipogenesis, and vice verse(21, 22). Bone mesenchymal stem cells (bMSCs) can be directed to either adipocyte or osteocye. One of main pathogenies about osteoporosis is more adipocyte originated from bMSCs (23). PPARγ is a well-known adipogenic transcriptional regulator. Agonist of PPARγ a serial of small molecules, which has been used as efficacious drugs for the treatment of diabetes mellitus, such as rosiglitazone and pioglitazone(24). However, one of the most notorious adverse effect of these drug is osteoporosis(25, 26). For this reason, PPARγ agonist usually prescribed for the treatment of diabetes mellitus combing with bisphosphate, such as alendronate(27). Based on these evidences, decreasing the capability of adipogenesis in bMSCs may an efficient way to increase the amount of bone.

BMPs is a serial of secretary cytokines, and the osteogenic potential of BMP was first found and demonstrated by Marshall Urist in 1965(28). Because the excellent osteoblastic property of some BMPs, such as BMP2 and BMP7, it has been used for the treatment of bone defect, non-uion, and spinal fusion(29, 30). BMP9 is another member of BMPs, which has been demonstrated processing stronger osteogenic potential than that of BMP2 or BMP7 (2). For this reason, BMP9 maybe an excellent alternate for BMP2 or BMP7 to treat the related bone diseases. Although PPARγ is a classic adipogenic factor, it was still up-regulated by BMP9 and great amount of adipocytes were occurred in the BMP9-induced bone masses. However, PPARγ knockdown decreased the osteogenic ability of BMP9, instead of potentiating this effect(6). This evidence suggested that PPARγ is critical not only for adipogenesis, but also for osteogenesis; the osteogenic potential may be enhanced only if the adipogenic process was inhibited specifically.

As above mentioned, BMP9 up-regulated the well-known adipogenic marker, such as PPARγ. However, the osteoblastic ability of BMP9 was also reduced when PPARγ knockdown. This evidence suggested that PPARγ may also play an important role in osteogenesis. The adipogenic commitment of progenitor cells are controlled by various factors, such as PPARγ, TRAF4 and Leptin(31–33). To date, it remains unclear whether there exist any regulators which are exclusive for adipogenesis, no any effect on osteogenesis. Lox, also named as protein lysine 6-oxidase, can converse lysine or lysine residue to aldehyde and promote the physicological cross-link process of the matrix protein, which is very important for the mature of connective tissue(10, 11). It was reported that Lox plays an important role in the lineage

commitment of adipocyte during the development(34). Besides, the abnormal level of Lox is associated with the biological behavior of cancer cells, such as metastasis(14). For this reason, Lox may affect the osteogenesis process of progenitor cells. It was reported that Lox can promote adipogenesis through inhibiting the signal of FGF-2. Lox promoted the adipogenic transcriptional factors, such as PPARγ and CCAAT enhancer binding protein (C/EBP) α in 3T3-L1 cells(34). Usually, the commitment of progenitor cells between osteogenesis and adipogenesis is mutual exclusive. Hence, Lox may also participate in the regulation of osteoblastic differentiation, but its role in the osteoblastic differentiation keeps controversial. It was reported that over-expression of Lox promote the calcification in vascular smooth muscle cells, and inhibition or knockdown of Lox almost diminished the calcification(35). Conversely, it was reported that inhibition of Lox greatly enhances the osteoblastic differentiation induced by BMP4 in MSCs(36). The converse effects of Lox on calcification or mineralization may contribute to the different of cell types, and context. In this study, we found that inhibition or knockdown of Lox promoted the osteoblastic markers or bone masses induced by BMP9 in 3T3-L1 cells. These evidences supported that Lox may function as a switcher between adipogenesis and osteogenesis, and its function may different greatly from other factors greatly, such as PPARγ. However, the exact mechanism through which Lox control the osteogenic and adipogenic commitment keeps unclear in MSCs.

The osteogenic process duing development is well-regulated by various cytokines or signals harmoniously, such as Wnt, FGF, IGF, and TGF-β (37). An expanding evidences support that moderately activate Wnt/β-catenin signal can promote the development of bone or keep the normal bone density from osteoporosis(38). Tang et al reported Wnt/β-catenin can enhance the osteogenic potential of BMP9, and knockdown of β–catenin obviously reduce this function of BMP9 in MSCs. Accordingly, BMP9 increased the activity of Wnt/β-catenin signal apparently(39). Our previous study demonstrated that all-trans retinoic acid can strengthen the osteogenesis induced by BMP9 through increasing the activation of Wnt/β-catenin signal(40). Therefore, Wnt/β-catenin is very important for osteoblastic differentiation. However, how the activity of Wnt/β-catenin signal is regulated by BMP9 or other osteogenic factors remains unclear and need to be uncovered further. Our data showed that either inhibition or knockdown of Lox can increase the BMP9-induced osteoblastic markers, which suggested that Lox may also be involved in the function of BMP9 to regulate the activity of Wnt/β-catenin signal. Our data showed that inhibition of Lox potentiates the effect of BMP9 on increasing the level of β-catenin in nucleus. BMP9 increases the protein level of sclerostin, and this effect of BMP9 was reduced by combining with Lox specific inhibitor. Herein, the effect of BMP9 on the activity of Wnt/β-catenin signal may be mediated by Lox partly.

It was reported that hypoxia-inducible factor-1α (HIF-1α) can promote the epithelial-to-mesenchymal transition through activating Lox in paraquat-induced pulmonary fibrosis(41), and HIF-1α and Lox can be regulated mutually to promote the growth of tumor cells(42). Meanwhile, Hu et al reported that BMP9 can up-regulate HIF-1α to increasing the angiogenic signal which can synergistically enhance the osteogenic capability of BMP9(43). These evidences suggested that HIF-1α may be involved in the regulatory effect of Lox on the osteoblastic inducing function of BMP9. We found that HIF-1α can be increased by BMP9 in 3T3-L1 cells, which was enhanced by the inhibition or knockdown of Lox but reduced apparently by

over-expressing Lox. Based on other reports, our data also suggested that there may exist a regulatory loop between Lox and HIF-1α, and HIF-1α may be involved in mediating the regulatory effect of Lox on the osteoblastic induction ability of BMP9. It was reported that HIF-1α can activate Wnt/β-catenin signal pathway through regulating BCL9 expression in hepatocellular carcinoma(44). Hence, the effect of Lox on BMP9-increased Wnt/β-catenin signal may be carried out through regulating HIF-1α. We found that the protein level of SOST can be increased by BMP9, but reduced by combining the inhibitor of Lox. Correspondingly, the total protein level of β-catenin was elevated by BMP9. The effect of BMP9 on β-catenin was enhanced by inhibiting Lox, which was reduced notably by silencing HIF-1α. These data suggested that the effect of Lox on Wnt/β-catenin signal may be mediated partially through regulating the expression of HIF-1α during the osteoblastic commitment process induced by BMP9 in MSCs.

Summary, our findings indicated that the effect of Lox on BMP9's osteogenic potential may be mediated through regulating Wnt/β-catenin signal via sclerostin negatively. Meanwhile, our finding also suggested that disturbing the function of Lox may be another strategy to treat the bone related diseases, such as osteoporosis. However, the exact molecular mechanism through which Lox affects Wnt/β-catenin signal need to be further analyzed.

Abbreviation

BMP: Bone morphogenetic protein; Runx2: Runt-related transcription factor 2;OPN: Osteopotin; GAPDH: glyceraldehyde phosphate dehydrogenase; HIF-1α: Hypoxia-inducible factor 1α; bMSCs: Bone mesenchymal stem cells; μ-CT: micro-Computed Tomography; RIPA: Radio immunoprecipitation assay; cDNA: Complementary deoxyribonucleic acid ; NBT: Nitro Blue tetrazolium; BCIP: 5-Brom-4-chlor-3-indoxylphosphat; SOST: sclerostin; TGF-β: Transforming growth factor β; ECL: Enhanced chemiluminescence; SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Abbreviations

BMP: Bone morphogenetic protein; Runx2Runt-related transcription factor 2OPN: Osteopotin; GAPDH: glyceraldehyde phosphate dehydrogenase; HIF-1α: Hypoxia-inducible factor 1α; bMSCs: Bone mesenchymal stem cells; μ-CT: micro-Computed Tomography; RIPA: Radio immunoprecipitation assay; cDNA: Complementary deoxyribonucleic acid ; NBT: Nitro Blue tetrazolium; BCIP: 5-Brom-4-chlor-3-indoxylphosphat; SOST: sclerostin; TGF-β: Transforming growth factor β; ECL: Enhanced chemiluminescence; SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Declarations

Acknowledgement

Authors thank professor T.C. He (Medical center of the University of Chicago) for his generous provision of BMP9 and HIF-1α recombinant adenovirus.

Authors contributions

YD and BCH designed the study. YD, JH, YXD, YYY, HHL, XTY and JZ performed the experiments. YD, BCH and YXS prepared the manuscript.

Funding

This study was funded by the National Key Research and Development Program of China (2016YFC1000803), the National Natural Science Foundation of China (NSFC, 81572226) and Chongqing Health and Family Planning Commission (ZY201702104).

Availability of data and materials

The data and material supporting the conclusions of this article are available upon request.

Ethics statement

This study was approved by the ethics committee of Chongqing Medical University and performed in accordance with the Declaration of Helsinki.

Consent for publication

It's not applicable.

Conflict of interest

Authors declare that they have no conflict of interest.

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Figures

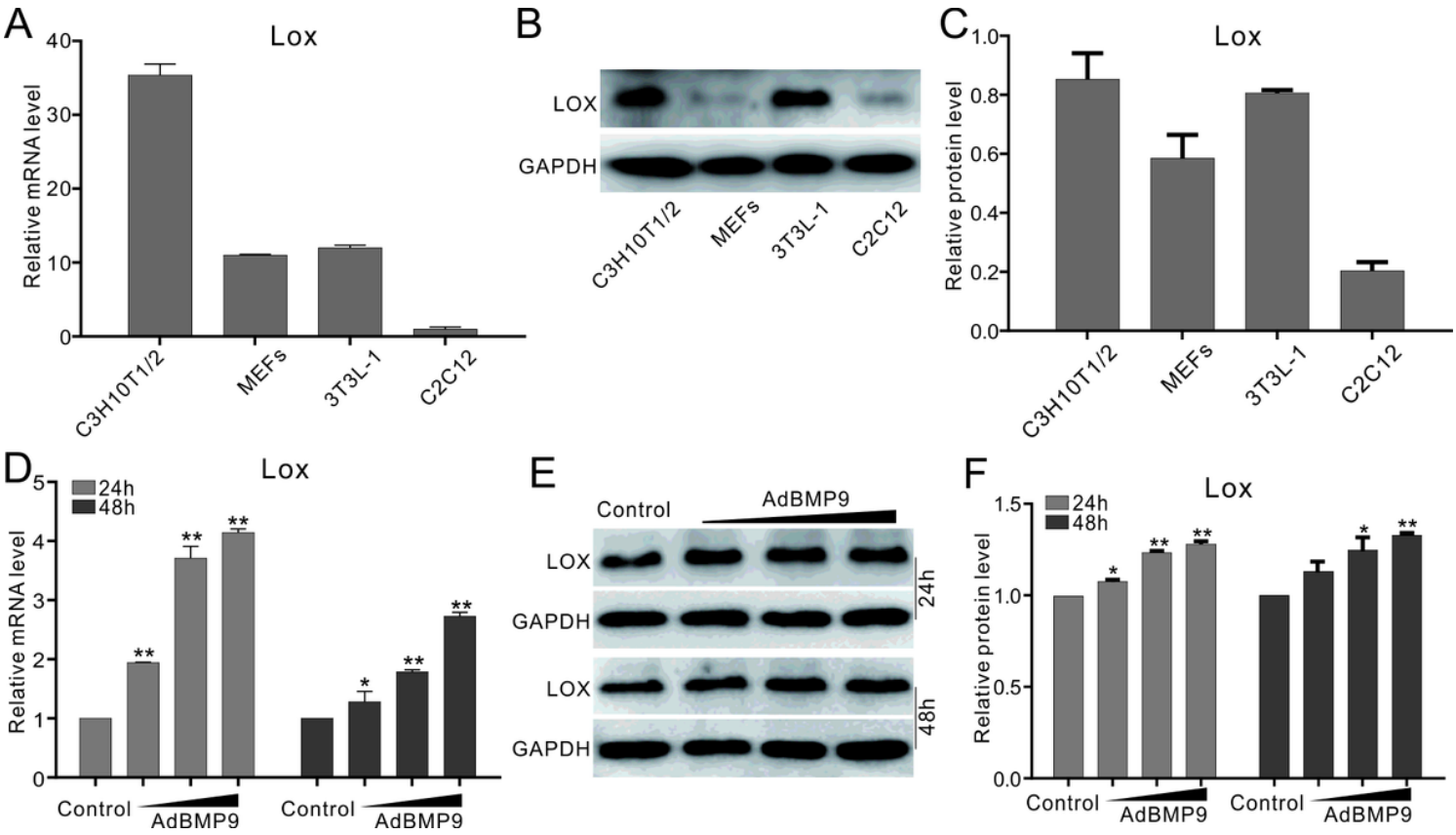

Figure 1

BMP9 affects the expression of lox in multiple progenitor cells (A) Real-time PCR analysis results show the mRNA expression level of Lox in C3H10T1/2, MEFs, 3T3-L1 and C2C12 cells. (B) Western blot assay results show the protein level of Lox in C3H10T1/2, MEFs, 3T3-L1 and C2C12 cells. (C) Quantitative results of western blot assay show the level of Lox in C3H10T1/2, MEFs, 3T3-L1 and C2C12 cells. (D) Real-time PCR assay results show the effect of BMP9 on the mRNA level of Lox in 3T3-L1 cells. (E) Western blot analysis results show the effect of BMP9 on the protein level of Lox in 3T3-L1 cells. (F) Quantitative results of western blot analysis show the effect of BMP9 on the level of Lox in 3T3-L1 cells. ("*" $p<0.05$, "**" $p<0.01$ vs control).

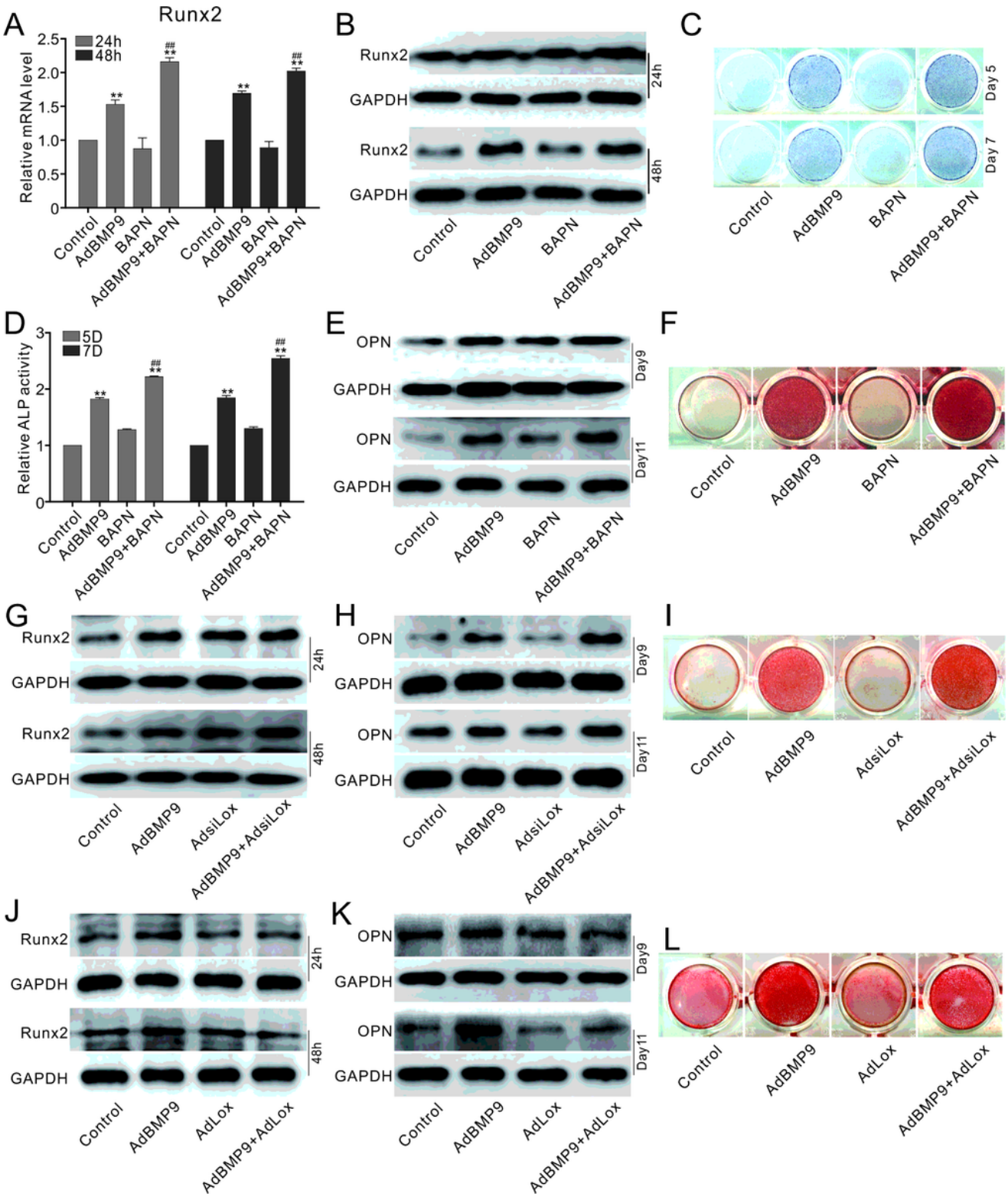

Figure 2

Lox affects the osteogenic markers induced by BMP9 in 3T3-L1 cells (A) Real-time PCR analysis show the effect of BMP9 and/or BAPN on the mRNA level of Runx2 in 3T3-L1 cells. (B) Western blot analysis show the effect of BMP9 and/or BAPN on the protein level of Runx2 in 3T3-L1 cells. (C) ALP staining show the effect of BMP9 and/or BAPN on the ALP activities in 3T3-L1 cells. (D) Quantitative results of ALP staining show the effect of BMP9 and/or BAPN on the ALP activities in 3T3-L1 cells. (E) Western blot

analysis show the effect of BMP9 and/or BAPN on the protein level of OPN in 3T3-L1 cells. (F) Alizarin Red staining show the effect of BMP9 and/or BAPN on matrix mineralization in 3T3-L1 cells. (G) Western blot analysis show the effect of BMP9 and/or Lox knockdown on the protein level of Runx2 in 3T3-L1 cells. (H) Western blot analysis show the effect of BMP9 and/or Lox knockdown on the protein level of OPN in 3T3-L1 cells. (I) Alizarin Red staining show the effect of BMP9 and/or Lox knockdown on matrix mineralization in 3T3-L1 cells. (J) Western blot analysis show the effect of BMP9 and/or Lox on the protein level of Runx2 in 3T3-L1 cells. (K) Western blot analysis show the effect of BMP9 and/or Lox knockdown on protein level of OPN in 3T3-L1 cells. (L) Alizarin Red staining show the effect of BMP9 and/or Lox on matrix mineralization in 3T3-L1 cells. (BAPN：β-aminopropionitrile，inhibitor of Lox, the concentration is 200 μM. “**” p<0.01 vs control; “##” p<0.01 vs BMP9 treated group).

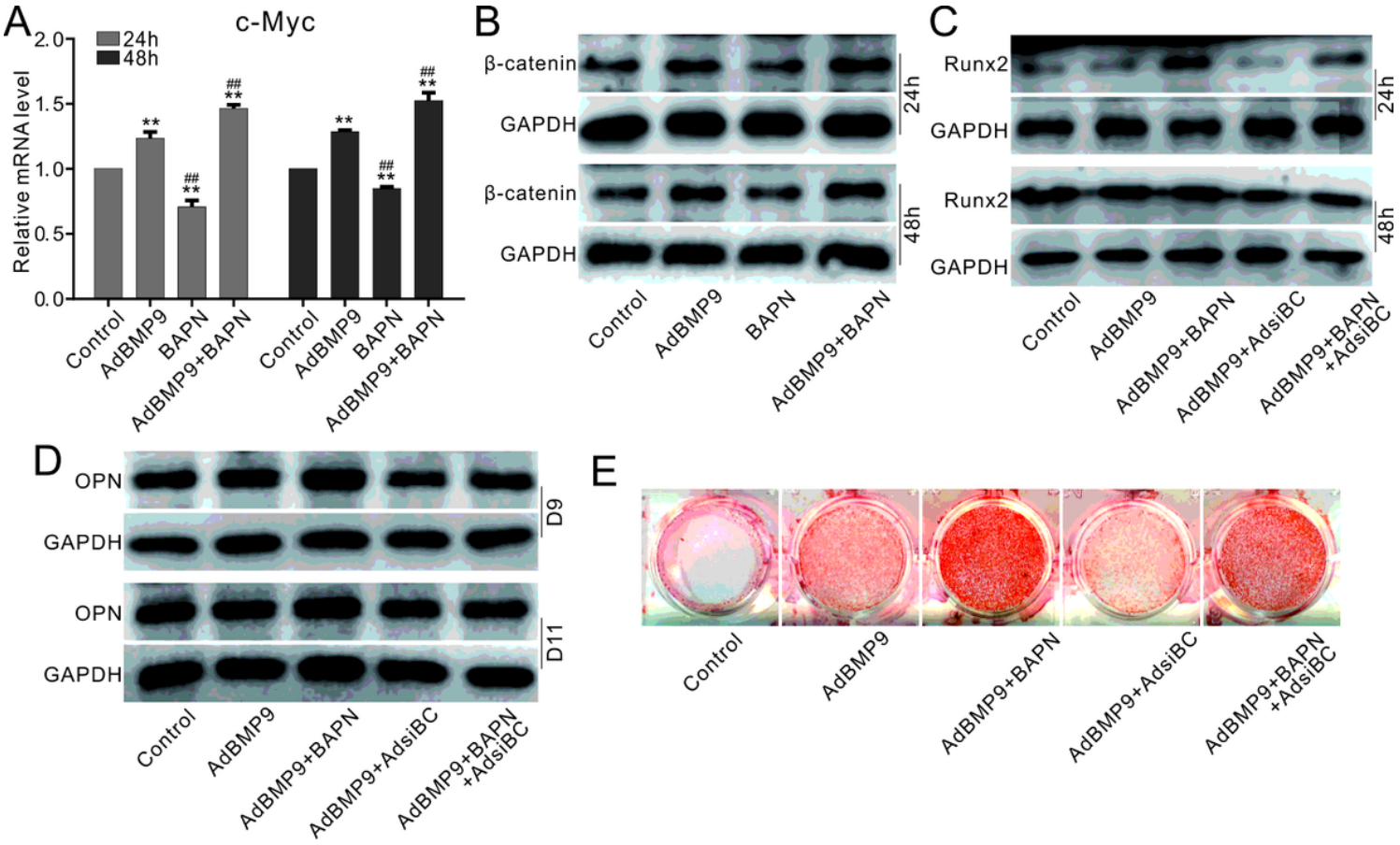

Figure 3

Effect of Lox and/or Wnt/β-catenin on the osteoblastic markers induced by BMP9 in 3T3-L1 cells (A) Real-time PCR analysis show the effect of BMP9 and/or BAPN on the mRNA level of c-Myc in 3T3-L1 cells. (B) Western blot analysis show the effect of BMP9 and/or BAPN on the protein level of β-catenin in 3T3-L1 cells. (C) Western blot analysis show the effect of BMP9，BAPN and/or β-catenin knockdown on the protein level of Runx2 in 3T3-L1 cells. (D) Western blot analysis show the effect of BMP9，BAPN and/or β-catenin knockdown on the protein level of OPN in 3T3-L1 cells. (E) Alizarin Red staining show the effect of BMP9，BAPN and/or β-catenin knockdown on the mineralization in 3T3-L1 cells. (BAPN：β-aminopropionitrile，inhibitor of Lox, the concentration is 200 μM. “**” p<0.01 vs control; “##” p<0.01 vs BMP9 treated group).

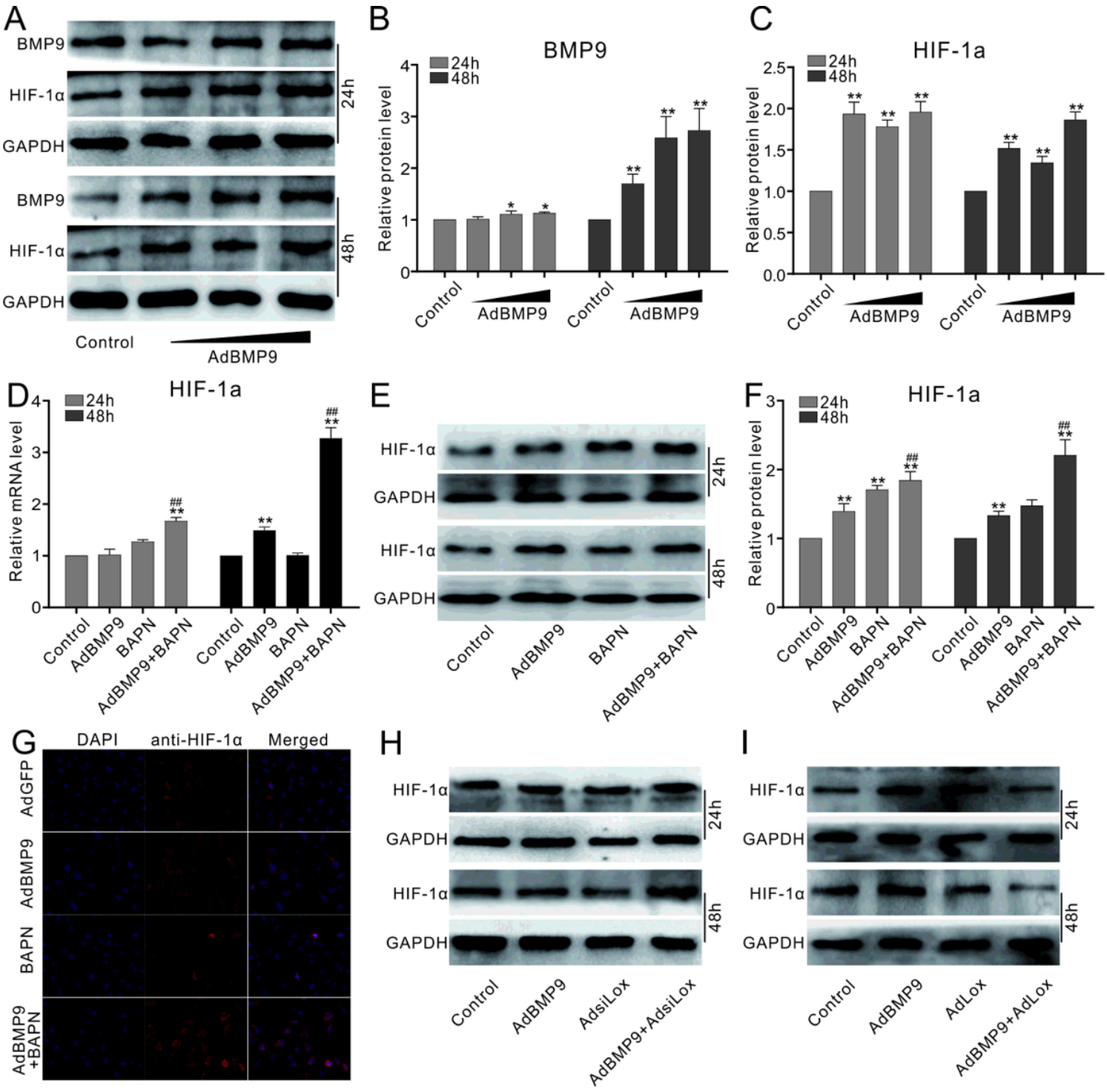

Figure 4

BMP9 and/or Lox affects the expression of HIF-1α in 3T3-L1 cells (A) Western blot analysis show the effect of BMP9 and/or Lox on the protein level of HIF-1α in 3T3-L1 cells. (B) Quantitative results of western blot show the effect of recombinant adenovirus of BMP9 the protein level of BMP9 in 3T3-L1 cells. (C) Quantitative results of western blot show the effect of BMP9 the protein level of HIF-1α in 3T3-L1 cells. (D) Real-time PCR analysis show the effect of BMP9 and/or BAPN on the mRNA level of HIF-1α in 3T3-L1 cells. (E) Western blot analysis show the effect of BMP9 and/or BAPN on the protein level of HIF-1α in 3T3-L1 cells. (F) Quantitative results of western blot analysis show the effect of BMP9 and/or

BAPN on the protein level of HIF-1α in 3T3-L1 cells. (G) Immunofluorescent staining show the effect of BMP9 and/or BAPN on the protein level of HIF-1α in 3T3-L1 cells. (H) Western blot analysis show the effect of BMP9 and/or Lox knockdown on the protein level of HIF-1α in 3T3-L1 cells. (E) Western blot analysis show the effect of BMP9 and/or Lox on the protein level of HIF-1α in 3T3-L1 cells. (BAPN：β-aminopropionitrile，inhibitor of Lox, the concentration is 200 μM. “*” p<0.05 vs control, “**” p<0.01 vs control; “##” p<0.01 vs BMP9 treated group).

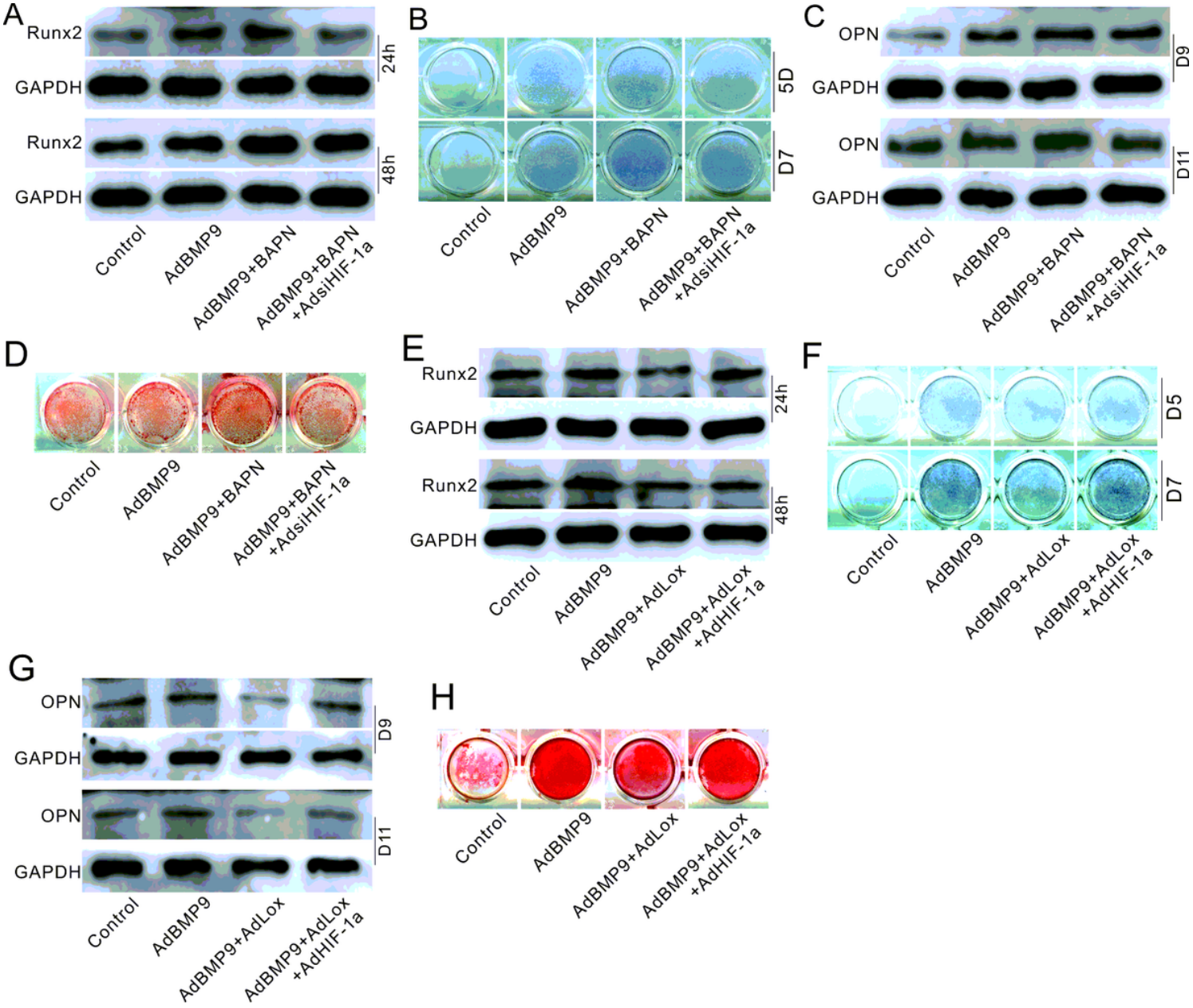

Figure 5

Lox and/or HIF-1α affects the osteoblastic markers induced by BMP9 in 3T3-L1 cells (A) Western blot analysis show the effect of BMP9、BAPN and/or HIF-1α knockdown on the protein level of Runx2 in 3T3-L1 cells. (B) ALP staining show the effect of BMP9、BAPN and/or HIF-1α knockdown on the protein level of ALP activities in 3T3-L1 cells. (C) Western blot analysis show the effect of BMP9、BAPN and/or HIF-1α knockdown on the protein level of OPN in 3T3-L1 cells. (D) Alizarin Red staining show the effect of

BMP9、BAPN and/or HIF-1α knockdown on the mineralization in 3T3-L1 cells. (E) Western blot analysis show the effect of BMP9、Lox and/or HIF-1α on the protein level of Runx2 in 3T3-L1 cells. (F) ALP staining show the effect of BMP9、Lox and/or HIF-1α on the protein level of ALP activities in 3T3-L1 cells. (G) Western blot analysis show the effect of BMP9、Lox and/or HIF-1α on the protein level of OPN in 3T3-L1 cells. (H) Alizarin Red staining show the effect of BMP9、Lox and/or HIF-1α on the mineralization in 3T3-L1 cells. (BAPN、β-aminopropionitrile、inhibitor of Lox, the concentration is 200 μM)

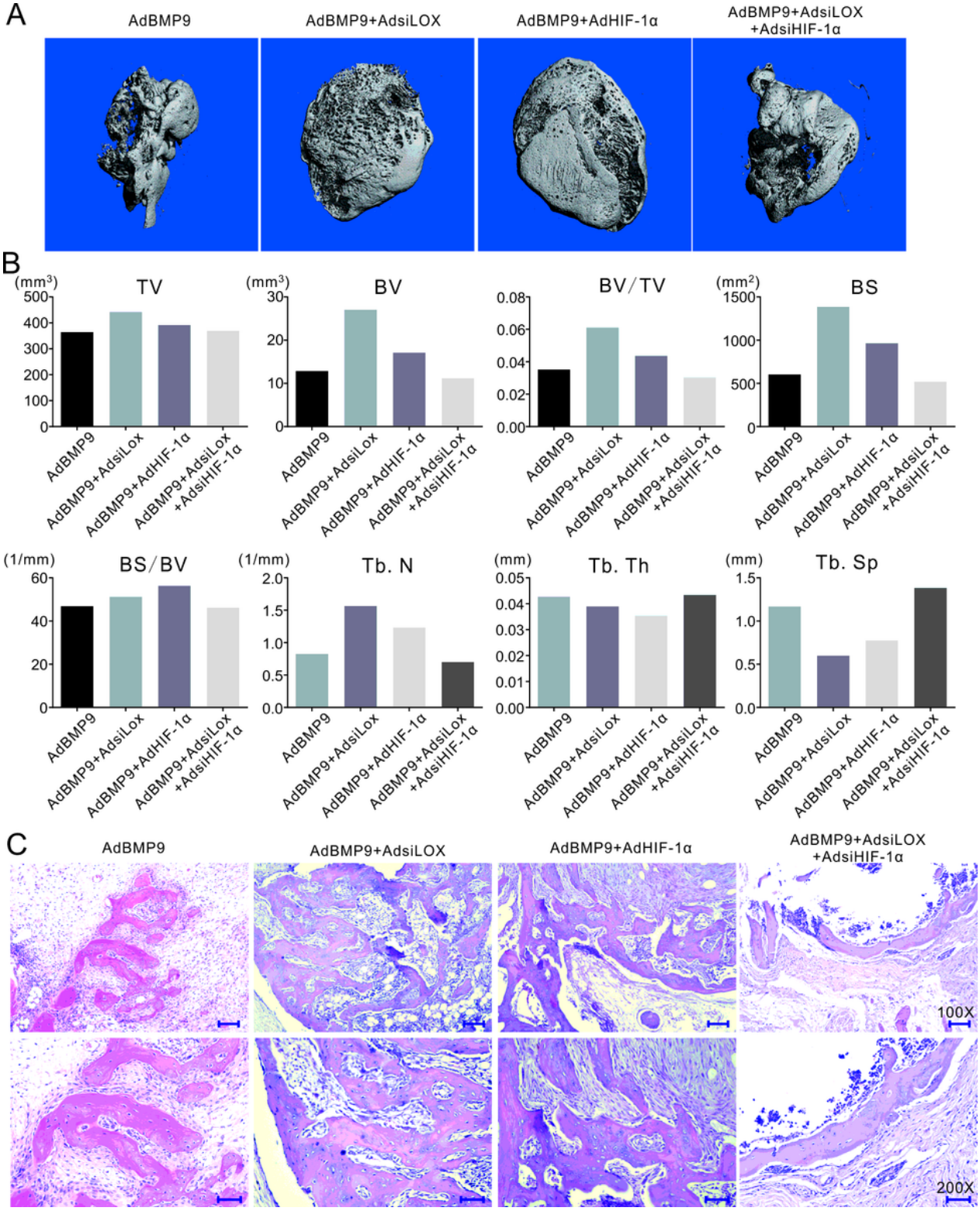

Figure 6

Lox and/or HIF-1α affects the bone formation induced by BMP9 in 3T3-L1 cells (A) 3D-reconstruction results of μ-CT scanning show the effect of BMP9, Lox knockdown, HIF-1α and/or HIF-1α knockdown on bone formation in 3T3-L1 cells. (B) Quantitative results of u-CT scanning show the effect of BMP9, Lox knockdown, HIF-1α and/or HIF-1α knockdown on bone formation in 3T3-L1 cells (TV: total volume, BV bone volume, BS: bone surface area, Tb.N: trabecular number, Tb.Th: trabecular thickness, Tb.Sp: trabecular separation). (C) H&E staining show the effect of BMP9, Lox knockdown, HIF-1α and/or HIF-1α knockdown on bone formation in 3T3-L1 cells (Scale bar: 200 μm for upper panel, 50 μm for lower panel)

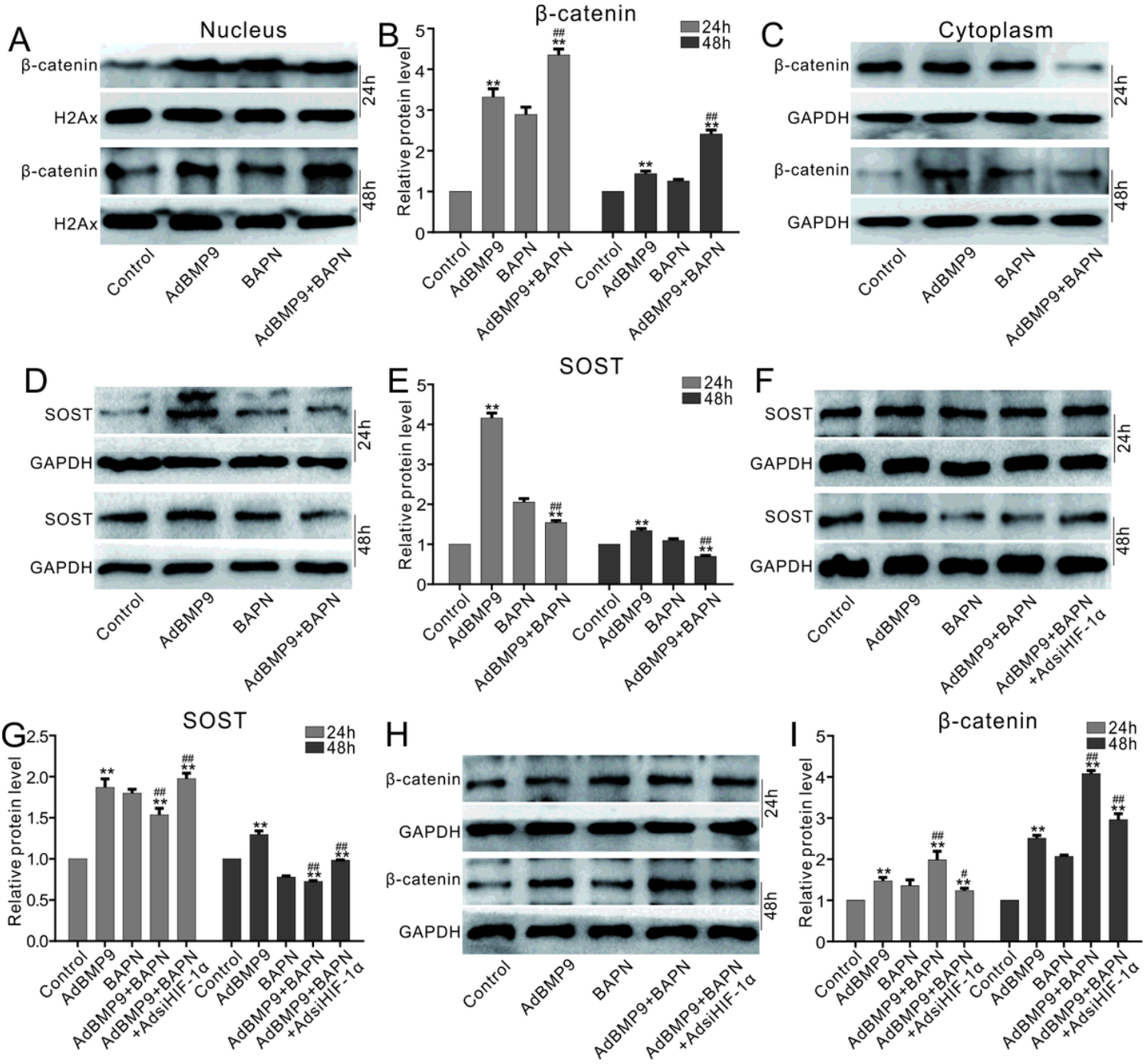

Figure 7

Lox and/or HIF-1α affects the effect of BMP9 on Wnt/β-catenin pathway (A) Western blot analysis show the effect of BMP9 and/or BAPN on the level of β-catenin in nucleus in 3T3-L1 cells. (B) Quantitative of

western blot analysis show the effect of BMP9 and/or BAPN on the level of β-catenin in nucleus in 3T3-L1 cells. (C) Western blot analysis show the effect of BMP9 and/or BAPN on the level of β-catenin in cytoplasm in 3T3-L1 cells. (D) Western blot analysis show the effect of BMP9 and/or BAPN on the level of SOST in 3T3-L1 cells. (E) Quantitative of western blot analysis show the effect of BMP9 and/or BAPN on the level of SOST in 3T3-L1 cells. (F) Western blot analysis show the effect of BMP9、BAPN and/or HIF-1α knockdown on the level of SOST in 3T3-L1 cells. (G) Quantitative of western blot analysis show the effect of BMP9、BAPN and/or HIF-1α knockdown on the level of SOST in 3T3-L1 cells. (H) Western blot analysis show the effect of BMP9、BAPN and/or HIF-1α knockdown on the level of β-catenin in 3T3-L1 cells. (I) Quantitative of western blot analysis show the effect of BMP9、BAPN and/or HIF-1α knockdown on the level of β-catenin in 3T3-L1 cells. (BAPN、β-aminopropionitrile、inhibitor of Lox, the concentration is 200 μM. “**” $p<0.01$ vs control; “##” $p<0.01$ vs BMP9 treated group).