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Smad4 mediated BMP2 signal is essential for the regulation of GATA4 and Nkx2.5 by affecting the histone H3 acetylation in H9c2 cells

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ABSTRACT

BMP2 signaling pathway plays critical roles during heart development, Smad4 encodes the only common Smad protein in mammals, which is a pivotal nuclear mediator. Our previous studies showed that BMP2 enhanced the expression of cardiac transcription factors in part by increasing histone H3 acetylation. In the present study, we tested the hypothesis that Smad4 mediated BMP2 signaling pathway is essential for the expression of cardiac core transcription factors by affecting the histone H3 acetylation. We successfully constructed a lentivirus-mediated short hairpin RNA interference vector targeting Smad4 (Lv-Smad4) in rat H9c2 embryonic cardiac myocytes (H9c2 cells) and demonstrated that it suppressed the expression of the Smad4 gene. Cultured H9c2 cells were transfected with recombinant adenoviruses expressing human BMP2 (AdBMP2) with or without Lv-Smad4. Quantitative real-time RT-PCR analysis showed that knocking down of Smad4 substantially inhibited both AdBMP2-induced and basal expression levels of cardiac transcription factors GATA4 and Nkx2.5, but not MEF2c and Tbx5. Similarly, chromatin immunoprecipitation (ChIP) analysis showed that knocking down of Smad4 inhibited both AdBMP2-induced and basal histone H3 acetylation levels in the promoter regions of GATA4 and Nkx2.5, but not of Tbx5 and MEF2c. In addition, Lv-Smad4 selectively suppressed AdBMP2-induced expression of HAT p300, but not of HAT GCN5 in H9c2 cells. The data indicated that inhibition of Smad4 diminished both AdBMP2 induced and basal histone acetylation levels in the promoter regions of GATA4 and Nkx2.5, suggesting that Smad4 mediated BMP2 signaling pathway was essential for the regulation of GATA4 and Nkx2.5 by affecting the histone H3 acetylation in H9c2 cells.

1. Introduction

Bone morphogenetic protein (BMP)-2 is an important and evolutionally conserved signaling molecule during cardiogenesis [1–3]. The important role of BMP2 in heart development is the most considered to be due to its ability to regulate the expression of cardiac core transcription factors. Our previous study showed that BMP2 induced hyperacetylation of histone H3 in the promoter regions of GATA4 and Nkx2.5 and promoted the gene expression. However, the upstream regulators are yet to be identified.

Smads perform important functions in the intracellular signaling of BMP. BMP-mediated activation of type I receptor and type II serine/threonine kinase receptors stimulates the phosphorylation of Smad1, Smad5 and Smad8 and subsequent heteromeric complex formation with Smad4. The Smad complexes translocate into the nucleus where they, in co-operation with co-activators or co-repressors, regulate gene expression. Smad4 encodes the only common Smad protein in mammals, which is a critical nuclear mediator in mediating BMP signaling pathway [4–6]. In addition to promoting Smad-mediated transcription, BMP ligands may also stimulate other molecules through "non-canonical" kinase pathways [7–9].

Deletion of Smad4 led to embryonic lethality, and embryos exhibited severe heart defects. In addition, deletion of Smad4 resulted in perturbation of BMP ligand expression and defects in the expression of some cardiac transcription factor genes such as Nkx2.5, GATA4, and MEF2c [10–13]. However, the underlying mechanism remains largely unknown.

In the present study, we hypothesize that Smad4 mediated BMP2 signaling pathway is essential for the expression of cardiac core transcription factors by affecting the histone H3 acetylation. To examine the role of Smad4 in mediating BMP2 induced hyperacetylation of histone H3 in the promoter regions of cardiac core transcriptional factors, we used the lentivirus-based vector to inhibit

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the Smad4 expression. We observed that inhibition of Smad4 diminished both BMP2 induced and basal histone acetylation levels in the promoter regions of GATA4 and Nkx2.5.

2. Materials and methods

2.1. Reagents

Recombinant adenoviruses expressing human BMP2 (AdBMP2), control adenoviruses expressing green fluorescent protein (AdGFP), and H9c2 cell lines were donated from the Molecular Oncology Laboratory at the University of Chicago Medical Center. Lentivirus-mediated Smad4-specific shRNA vector expressing the puromycin resistance gene in H9c2 cells (Lv-Smad4) and Lentivirus containing NC-shRNA (NC, as negative controls) vector was synthesized and packaged by hanbio biotechnology (shanghai) co., ltd.

2.2. Culture and treatment of H9c2 cell lines

H9c2 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM)/high glucose (Thermo, USA) containing 10% fetal bovine serum (FBS) (Hyclone, USA) and 1% penicillin (Thermo, Waltham, MA, USA) humidified with 5% CO_2 air at 37 °C. When reached 80% confluence, the cells were transfected with AdBMP2 with or without exposure to Lv-Smad4. The control cells were transfected with AdGFP or Lv-NC. Lv-Smad4 was transfected with H9c2 cells and then establishes stable cell lines that knock down the Smad4 gene using 2ug/ml puromycin.

2.3. Quantitative real-time RT-PCR

Total RNA was extracted from H9c2 cells using a RNA extract kit (Bioteck, Beijing, China). Single-strand cDNA was reverse transcribed at 42 °C for 15 min from 300 to 800 ng RNA using a PrimeScript RT reagent Kit (TaKaRa, Biotechnology, Dalian, China). The cDNA was amplified with gene-specific primers (TaKaRa, Biotechnology, Dalian, China) and SYBR Green RealMasterMix kit (Tiangen, Beijing, China). The primers' sequences for heart development-related genes and control genes were as follows. The annealing temperature was 54 °C for BMP2, 57 °C for GATA4 and Tbx5, 61 °C for Smad4, 65 °C for MEF2c, p300, GCN5, and β-actin, and 63.6 °C for Nkx2.5. The gene-specific primers were designed using the Primer-3 software as follows: for BMP2: 5′-GACATCCACTCCAC AAACGAGA-3′ (upper) and 5′-GTCATTCCACCCCACATCACT-3′ (lower); for GATA4: 5′-CAACTGCCAGACTACCACCAC-3′ (upper) and 5′-CCATGGAGCTTCATGTAGAGG-3′ (lower); for Nkx2.5: 5′-TC GCAGGCGCAGGTCTAT-3′ (upper) and 5′-ACACTTGTAGCGGCGGTTC T-3′(lower); for MEF2c: 5′-GCGAAAGTTCGGATTGATGAAGA-3′ (upper) and 5′-GTGGATGTCAGTGCTGGCGTA-3′ (lower); for Tbx5: 5′-CAAGGCAGGGAGGCAGATGTT-3′ (upper) and 5′-GCAGGCTCGGC TTTACCAGTT-3′ (lower); for p300: 5′-AGATTCAGAGGGCAGCAGAG AC-3′ (upper) and 5′-GCCATAGGAGGTGGGTTCATAC-3′ (lower); for GCN5: 5′-GGAAAGGAGAAGGGCAAGGAG-3′ (upper) and 5′-GT CAATGGGGAAGCGGATAAC-3′ (lower); for Smad4: 5′-CCTGGGTCC GTAGGTGGAATAG-3′ (upper) and 5′-CTTTGATGCTCTGTCTCGGGT AG-3′ (lower); for β-actin: 5′-GGAGATTACTGCCCTGGCTCCTA-3′ (upper) and 5′-GACTCATCGTACTCCTGCTTGCTG-3′ (lower). Relative mRNA expression was analyzed using the $2^{-\triangle\triangle Ct}$ method as described previously [14]. The values were normalized using β-actin as control genes.

2.4. Western blotting analysis

Nucleoproteins were extracted with a Nuclear Extract Kit (Keygen, Nanjing, China). Protein concentration was detected using a BCA protein assay kit (Thermo Scientific). The protein preparations were loaded on 15% Bis–Tris polyacrylamide gels for electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). After blocked with 5% nonfat milk in PBST (PBS plus 0.05% Tween 20) for 1 h, the PVDF membranes were exposed to rabbit monoclonal antibody for acetylated histone H3 (Ac-H3) (Millipore, Temecula, CA, USA, 1:500 dilution) or total histone H3 (Millipore, Temecula, CA, USA, 1:500 dilution) in PBST plus 3% nonfat milk and 0.05% Tween-20 at 4 °C overnight. HRP-conjugated goat anti-rabbit (Santa Cruz Biotechnology, CA, USA) was used as the secondary antibody. Protein bands were revealed with an Enhanced Chemiluminescence Luminal reagent (Millipore, USA) and analyzed with Quantity One Version 4.4 software (Bio-Rad, CA, USA). The density of the Ac-H3 band was normalized to histone H3 band in each sample.

2.5. Chromatin immunoprecipitation (ChIP) assay

The ChIP analysis was used to evaluate the level of histone H3 acetylation in the promoters of cardiac development-related transcription factors. ChIP experiments were performed using a ChIP assay kit (Millipore, MA, USA) following manufacturer's protocols. Proteins and DNA were cross-linked after fixing the cultured H9c2 with formaldehyde (37%, sigma, USA). DNAs were ultrasonically cut into small fragments ranged from 200 to 1000 bp. The protein-DNA complexes were precipitated using rabbit monoclonal antibody against Ac-H3. Antibody for RNA polymerase was used as a positive control and normal mouse IgG as a negative control. The protein-DNA complexes were also collected without antibody as input group (to show the total DNA in the samples). The DNA was obtained after removing the cross-link of proteins and DNA.

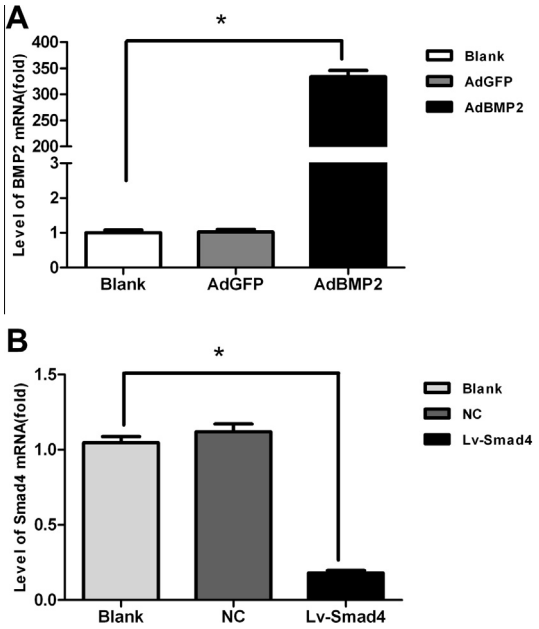

Fig. 1. (A) Expression of bone morphogenetic protein (BMP)-2 in H9c2 cells transfected with recombinant adenoviruses expressing human BMP2 (AdBMP2). Transfecting with AdBMP2 or control adenoviruses expressing green fluorescent protein (AdGFP) in H9c2 cells. The mRNA level of BMP2 was measured 48 h after transfection. Quantitative real-time PCR analysis showed the overexpression of BMP2 gene in the cells transfected with AdBMP2 (AdBMP2 group vs. blank group, $^{*}p < 0.01$). Fig. 1(B) Expression of Smad4 in H9c2 cells transfected with lentivirus-mediated Smad4-specific shRNA vector in H9c2 cells (Lv-Smad4). Transfecting with Lv-Smad4 and Lentivirus containing NC-shRNA (NC, as negative controls) vector in H9c2 cells. The mRNA level of Smad4 was measured after steadily transfection. Quantitative real-time PCR analysis showed the decrease in Smad4 gene in the cells transfected with Lv-Smad4 (Lv-Smad4 group vs. NC group, $^{*}p < 0.01$).

Specific primers were designed to determine the acetylation level of histone H3 in promoter regions of GATA4, MEF2c, Nkx2.5 and Tbx5 for quantitative real-time PCR analysis. The sequences of specific primers were as follows: for GATA4: 5′-ACTGACGCCGACTCCA AACTAAG-3′ (upper) and 5′-GTGTCCCTGTTCTCCCTGTAGC-3′ (lower); for Nkx2.5: 5′-TGGACAAAGCCGAGAGAGACG -3′ (upper) and 5′-CACTTGTAGCGGCGGTTCTG -3′ (lower); for MEF2c: 5′-CTTT CCAGGTTGGCTCTTACTCC-3′ (upper) and 5′-GCCTCCTCCTAACAAAG TGGGTA-3′ (lower); for Tbx5: 5′-GAGAGCCAGCCACGGAATC-3′ (upper) and 5′-TCTTCCCAGCCCTAAGCAATAC-3′ (lower). The annealing temperature was 65 °C for GATA4 and MEF2c, and 57 °C for Tbx5. Relative promoter precipitation level was analyzed using the $2^{-\Delta\Delta Ct}$ method as described previously [14]. The ChIP sample values were normalized to the input sample values.

2.6. Statistical analysis

Experiments were independently repeated at least three times with consistent results. All the data were expressed as mean ± SD and statistically analyzed using one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Lv-Smad4 and AdBMP2 were effectively transfected and expressed in H9c2 cells

To examine the expression of BMP2 in H9c2 cells after transfection with AdBMP2, quantitative real-time PCR assay was used to

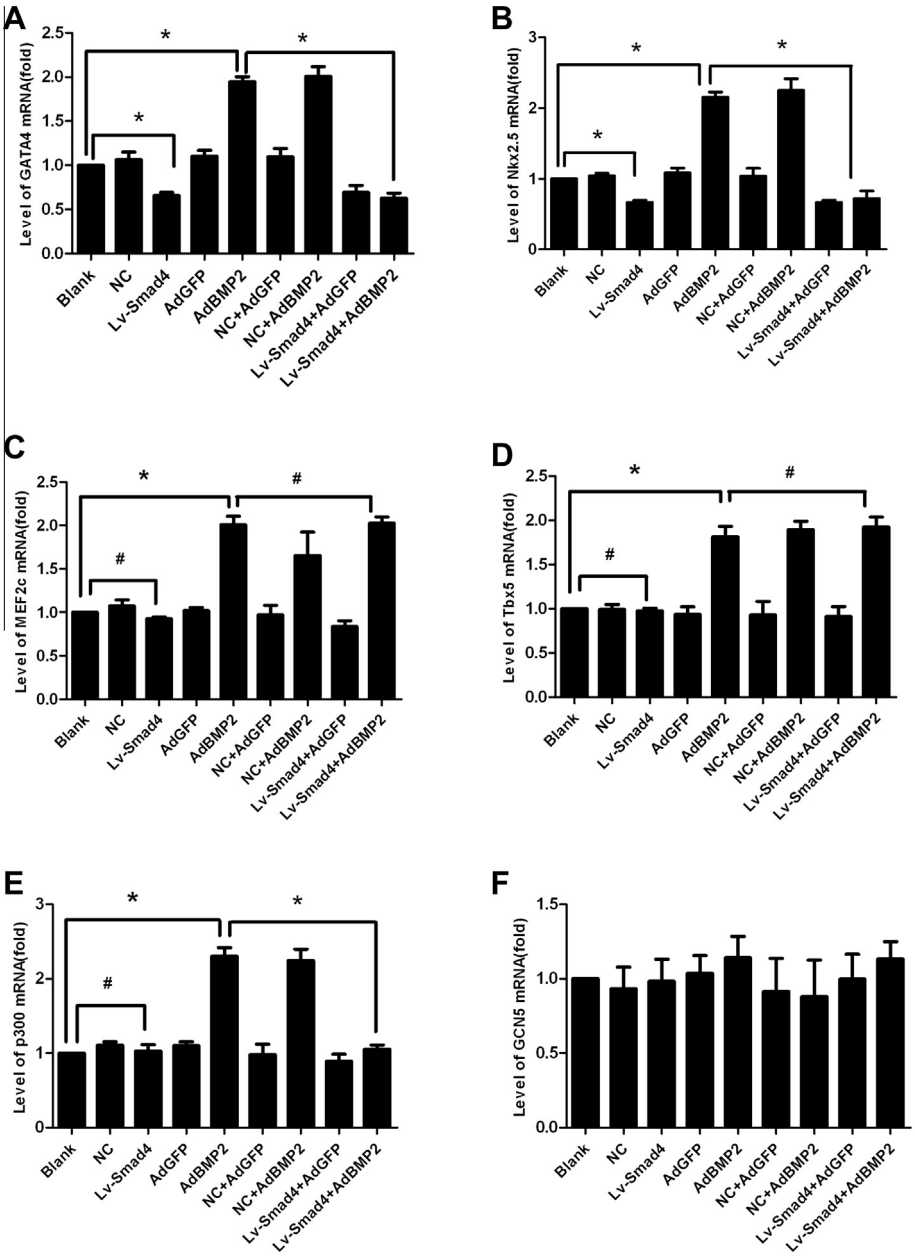

Fig. 2. Effect of inhibition of Smad4 on the mRNA levels of GATA4, Nkx2.5, MEF2c, Tbx5 and HATs p300 and GCN5 in H9c2 cells transfected with or without AdBMP2. (A, B and E) Quantitative real-time RT-PCR analysis showed that both AdBMP2-induced and basal expressions of GATA4, Nkx2.5 and HATs p300 were significantly inhibited by Lv-Smad4 (AdBMP2 + Lv-Smad4 group vs. AdBMP2 group, and Lv-Smad4 group vs. blank group). (C and D) Quantitative real-time RT-PCR analysis showed that AdBMP2-induced expression of MEF2c and Tbx5 were not significantly inhibited by Lv-Smad4 (AdBMP2 + Lv-Smad4 group vs. AdBMP2 group). (F) Neither Lv-Smad4 nor AdBMP2 significantly changed the mRNA level of GCN5 in the cells. $^{*}p < 0.05$.

detect the BMP2 mRNA level. We observed that BMP2 peak expression occurred 48 h after the transfection with 330 fold increase (AdBMP2 group vs. blank group, $^{*}p < 0.05$) as shown in Fig. 1A.

To detect the knockdown efficiency of Smad4 gene in H9c2 cells, quantitative real-time PCR assay was used to measure the Smad4 mRNA level. We observed that Smad4 was efficiently inactivated by 82.8% (Lv-Smad4 group vs. blank group, $^{*}p < 0.05$) indicating that the transcriptional expression of Smad4 was effectively inhibited with the vector. No significant change in the Smad4 mRNA level was observed in the cells transfected with the control vector as shown in Fig. 1B.

3.2. Lv-Smad4 selectively inhibited AdBMP2-induced expression of cardiac transcription factors GATA4 and Nkx2.5, but not MEF2c and Tbx5, and HATs subtype p300 but not GCN5 in H9c2 cells

The quantitative real-time RT-PCR analysis demonstrated that the mRNA levels of GATA4, Nkx2.5, MEF2c, Tbx5 and HATs subtype p300 were significantly increased in H9c2 cells overexpressing BMP2 as shown in Fig. 2 (AdBMP2 group vs. blank group, $^{*}p < 0.05$). Treatment of the cells with Lv-Smad4 significantly decreased AdBMP2-induced increase in the expression of GATA4 and Nkx2.5 (AdBMP2 group vs. AdBMP2 + Lv-Smad4 group $^{*}p < 0.05$).Similarly, the basal expression of GATA4 and Nkx2.5 was also decreased in H9c2 cells after Lv-Smad4 treatment (Lv-Smad4 group vs. blank group, $^{*}p < 0.05$). However, there was no significant change in the expression of MEF2c, and Tbx5 in the cells transfected with AdBMP2 after Lv-Smad4 treatment. There was no significant change in the expression of HATs subtype GCN5 in all of the groups.

3.3. Inhibition of Smad4 significantly decreased AdBMP2-induced global hyperacetylation of histone H3 in H9c2 cells

Western blotting analysis showed that histone H3 acetylation level was significantly increased in H9c2 cells overexpressing BMP2 as shown in Fig. 3 (AdBMP2 group vs. blank group, $p < 0.05$). The AdBMP2- induced histone H3 acetylation was significantly inhibited after Lv-Smad4 treatment (AdBMP2 group vs. AdBMP2 + Lv-Smad4 group $^{*}p < 0.05$), but the basal histone H3 acetylation level was not decreased in H9c2 cells after Lv-Smad4 treatment (Lv-Smad4 group vs. blank group, $^{\#}p > 0.05$).

3.4. Inhibition of Smad4 selectively decreased histone H3 acetylation level in GATA4 and Nkx2.5 promoter region but not MEF2c and Tbx5 in H9c2 cells transfected with AdBMP2

The ChIP and quantitative real-time PCR analyses showed that the promoter DNA quantities of GATA4 and Nkx2.5 were significantly increased in H9c2 cells overexpressing BMP2 (AdBMP2 group vs. blank group, $^{*}p < 0.05$) (Fig. 4). Similarly, both AdBMP2-induced and basal histone H3 acetylation levels in the promoter regions of GATA4 and Nkx2.5 were substantially inhibited in the cells treated with Lv-Smad4 (AdBMP2 + Lv-Smad4 group vs. AdBMP2 group, and Lv-Smad4 group vs. blank group $^{*}p < 0.05$).

4. Discussion

Heart is the first organ to form during the embryo development. Heart development is a complex process and controlled by a group of cardiac transcription factors such as GATA4, Nkx2.5, MEF2c and Tbx5 through a complex network of signaling pathways such as BMP2 signaling [15,16]. BMP2 mutant mice exhibit heart defects in the atrial septum and ventricular outflow tract, and deficient valve differentiation and cushion formation [12]. Several studies showed that BMP2 could induce ectopic expression of cardiac transcription factors such as Nkx2.5, and GATA4, suggesting that BMP2 is an inductive signaling molecule that is involved in heart development [17]. However, the molecular mechanism by which BMP2 regulates the cardiac transcription factors remains largely unknown. One previous study showed that BMP4 could suppress the expression of StAR by inhibiting histone H3 acetylation in the promoter regions of the gene in bovine granulosa cells [18]. Pan et al. suggested that BMP2 activated Sox9 gene transcription by affecting chromatin remodeling and histone modification in primary mouse embryo fibroblasts [13]. In the present study, we found that BMP2 induced hyperacetylation of histone H3 in the promoter regions of cardiac transcription factors GATA4 and Nkx2.5, since histone hyperacetylation is related to gene overexpression, thus enhanced the mRNA expression of GATA4 and Nkx2.5, which is consistent with our previous study [19].

In recent years, histone acetylation plays an important role in heart development [20]. Histone acetylation is a dynamic process that is regulated by histone acetylases (HATs) and histone deacetylases (HDACs). It regulates the state of chromatin to decide inhibition or activation of gene expression. However, the upstream mechanism of histone acetylation remains unclear. Cardiomyocyte differentiation is a process that requires the interaction of transcription factors such as GATA4, Nkx2.5, MEF2c, and Tbx5 (GNMT). Chromatin-modifying factors are associated with these transcriptional networks, and the epigenetic control of them and their targets by chromatin-modifying enzymes is involved in proper cardiac differentiation. Several HATs are known to regulate cardiac gene expression [21–23]. The E1A-associated nuclear protein p300 is most studied in heart development. A genetic knock-in study showed that the p300 is essential for cardiogenesis [24], while Homozygous p300 knockout mice exhibited reduced heart trabeculation and diminished expression of cardiac structural proteins

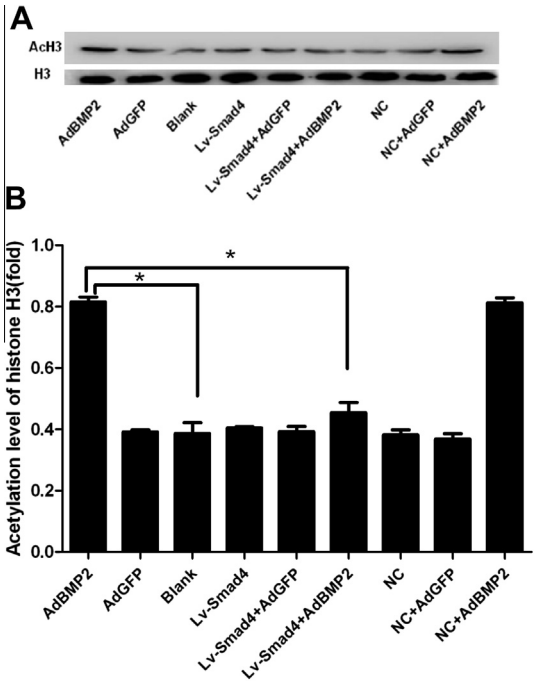

Fig. 3. Effect of Lv-Smad4 on global histone H3 acetylation in the chromatin in H9c2 cells transfected with or without AdBMP2. (A) Representative Western blotting bands of Ac-H3 (top) and H3 (bottom, as an endogenous control). (B) Summary data from three independent experiments for the ratio of Ac-H3 to H3, analysis showed that AdBMP2-induced hyperacetylation of histone H3 was significantly inhibited by Lv-Smad4 (Ad-BMP2 + Lv-Smad4 group vs. AdBMP2 group, AdBMP2 group vs. blank group). $^{*}p < 0.05$.

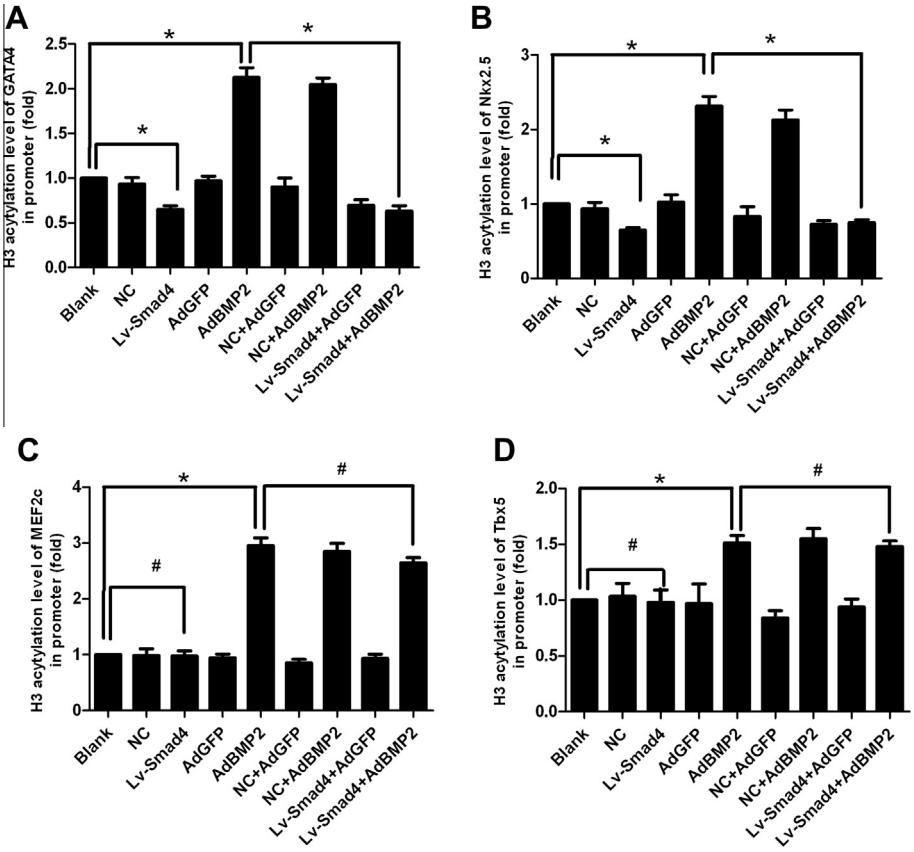

Fig. 4. Effect of Lv-Smad4 on Histone H3 acetylation in the promoter regions of GATA4, Nkx2.5, MEF2c and Tbx5 in H9c2 cells transfected with or without AdBMP2. The DNA quantity of transcription factors in the promoter region immunoprecipitated with acH3 antibody represented acetylation level of histone H3 in the gene promoter regions. (A and B) Chromatin immunoprecipitation (ChIP) and quantitative real-time PCR analyses showed that both AdBMP2-induced and basal acetylation levels of histone H3 in the promoter regions of GATA4 and Nkx2.5 were significantly inhibited by Lv-Smad4 (AdBMP2 + Lv-Smad4 group vs. AdBMP2 group, and Lv-Smad4 group vs. blank group). (C and D) Analysis showed that AdBMP2-induced hyperacetylation in the promoter regions of MEF2c and Tbx5 was not significantly inhibited by Lv-Smad4 (AdBMP2 + Lv-Smad4 group vs. AdBMP2 group, $^{\#}p > 0.05$).

such as α-MHC and α-actinin [20,21]. Our previous study showed that inhibition of p300-HAT results in a reduced histone acetylation and down-regulation of gene expression in cardiac myocytes [22]. In the present study, we observed that BMP2 promoted the expression of p300 and the hyperacetylation of GNMT in the promoter regions, therefore, p300 may be involved in the regulation of these transcriptional factors and we presumed that BMP2 signal is the upstream mechanism of regulated HATs such as p300.

Several studies showed that BMP signals are transducted through canonical Smad4 and also "non-canonical" kinase pathways such as MAPK, and Akt signal pathway [4,5]. Numerous studies have reported that Smad4 plays important roles in regulating the development of the cardiovascular system. Lanying Song et al showed that myocardial-specific inactivation of Smad4 caused heart failure and embryonic lethality at midgestation [11]. We hypothesize that Smad4 mediated BMP2 signal pathway is essential for the expression of cardiac core transcription factors by affecting the histone H3 acetylation and we knocked down the Smad4 gene and detected the gene expression and level of histone acetylation. Our results showed that inhibition of Smad4 decreased both BMP2-induced and basal expression levels of cardiac transcription factors GATA4 and Nkx2.5, but not MEF2c and Tbx5, Similarly, Lv-Smad4 inhibited both BMP2-induced and basal histone H3 acetylation levels in the promoter regions of GATA4 and Nkx2.5, but not of MEF2c and Tbx5.These data suggested that Smad4 is essential for the BMP2 induced GATA4 and Nkx2.5 expression and their hyperacetylation in the promoter regions. In addition, BMP2 induced hyperacetylation in the promoter regions of MEF2c and Tbx5 were not inhibited by the disruption of Smad4, suggesting that Smad4 was not necessary for the process.

In conclusion, our data demonstrate that Smad4 is essential for BMP2 induced GATA4 and Nkx2.5 expression and their hyperacetylation in the promoter regions, in addition, there may exists Smad4-independent pathway to regulate the BMP2 induced hyperacetylation in the promoter regions of MEF2c and Tbx5 in H9c2 cells.

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