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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.

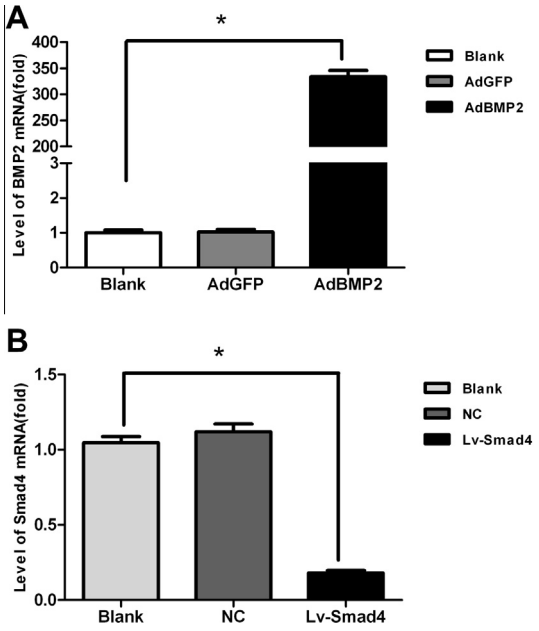

Fig. 1. (A) Expression of bone morphogenetic protein (BMP)-2 in H9c2 cells transfected with recombinant adenoviruses expressing human BMP2 (AdBMP2). Transfecting with AdBMP2 or control adenoviruses expressing green fluorescent protein (AdGFP) in H9c2 cells. The mRNA level of BMP2 was measured 48 h after 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, $^{*}p < 0.01$).

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