

# Bone morphogenetic protein-2 enhances the expression of cardiac transcription factors by increasing histone H3 acetylation in H9c2 cells

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**Abstract.** Bone morphogenetic protein (BMP)-2 induces the expression of cardiac transcription factors during early heart development, however, the underlying mechanisms for this are not clear. Our previous studies indicated that histone acetylation is critical in the regulation of cardiac gene expression. In the present study, the hypothesis that BMP2 enhances the expression of cardiac transcription factors by increasing histone H3 acetylation was tested. Cultured H9c2 rat embryonic cardiac myocytes were transfected with adenoviruses expressing human BMP2 (AdBMP2). Real-time RT-PCR, western blotting, chromatin immunoprecipitation (ChIP) and colorimetric assays were employed to determine gene expression, histone H3 acetylation levels and histone acetylase (HAT) activities. The mRNA expression levels of BMP2, GATA4, MEF2C and p300, but not of Tbx5 and GCN5, were significantly upregulated following transfection with AdBMP2. Similarly, the histone H3 acetylation levels were enhanced in the whole chromatin and in the promoter regions of GATA4 and MEF2C, but not Tbx5, in the transfected cells. The HAT activities were also enhanced. These results indicate that BMP2 is able to upregulate the expression of the cardiac transcription factors GATA4 and MEF2C, in part by increasing histone H3 acetylation in the promoter regions of these genes.

## Introduction

Numerous studies have shown that heart development is controlled by an evolutionarily-conserved network of tran-

scription factors (NK2, MEF2, GATA, Tbx and Hand) that connect signaling pathways with genes for muscle growth, patterning and contractility (1-5). In this network, GATA4, MEF2C and Tbx5 are the most significant members which play critical roles in cardiac cell growth and heart development (6). The disturbance of temporal-spatial expression or mutations in these cardiac transcription factors may cause congenital heart disease (1,7). However, the upstream regulation of these genes remains unclear.

Previous studies have revealed that bone morphogenetic protein (BMP) signaling is required for cardiogenesis (8-10). It has been reported that several BMP subtypes, including BMP2, 4, 6 and 7, are expressed in the developing heart (9) and that BMP2 is a major transcription factor among several other members of the BMP family. BMP2 plays a significant role during cushion and valve morphogenesis and has a persistent expression in the cushion mesenchyme from 13.5 to 16 days *post coitum* (dpc). The expression of BMP2 is also observed in the valve tissues of adult mice (11). BMP2 knockout mice die at 7.5-10.5 dpc and have defects in the heart during development (12). Clinically, patients with a heterozygous deletion of BMP2 may present tachycardia as a result of an abnormal connection between the atria and ventricles (13). Several studies have demonstrated that BMP2 is able to elicit ectopic expression of the early cardiac markers, including Nkx2.5 and GATA4, but not of Tbx5 (9,14). Schlange *et al* (15) reported that BMP2 was expressed dynamically during cardiac morphogenesis and regulated the expression of other cardiac transcription factors, including Nkx2.5 and GATA4, at varying time periods. However, the molecular mechanisms by which BMP2 regulates the cardiac transcription factors remain largely unknown.

In recent years, histone acetylation/deacetylation has been at the center of attention with regard to the control of gene expression. Acetylation of the conserved lysine residues in histone tails by histone acetylases (HATs) stimulates gene expression by neutralizing positive charges, resulting in the 'open' state of chromatin, while histone deacetylases (HDACs) promote chromatin condensation, causing a repression of gene expression (16-18). It has been reported that histone acetylation

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plays a significant role during the development of the heart by acting as a switch for the regulation of gene expression (18). In our previous study, sodium valproate (NaVP), an inhibitor of HDACs, was identified as being able to increase the expression of CHF1, Tbx5 and MEF2C, causing cardiac abnormalities in fetal mice (19). Alcohol-induced overexpression of heart development-related genes was also identified as associated with the upregulation of histone H3 lysine 9 acetylation (20). One study in rat bone marrow mesenchymal stem cells also showed that suberoylanilide hydroxamic acid (SAHA), a HDAC inhibitor, upregulated the expression of Nkx2.5, GATA4 and MEF2C in a dose-dependent manner (21). These studies indicate that histone acetylation regulates the expression of cardiac-specific genes and is essential for heart development.

In the present study, the histone H3 acetylation levels in the promoter regions of the cardiac-specific genes and the HAT activities in the cultured H9c2 rat embryonic cardiac myocytes overexpressing BMP2 were determined. The results indicate that BMP2 is able to enhance the expression of the cardiac transcription factors GATA4 and MEF2C, in part by increasing histone H3 acetylation in the promoter regions of these genes. The HAT p300 subtype may play an essential role in BMP2-induced histone hyperacetylation.

## Materials and methods

**Reagents.** The recombinant adenoviruses expressing human BMP2 (AdBMP2), the control adenoviruses expressing green fluorescent protein (AdGFP), the human embryonic kidney 293 cells and the H9c2 cells were kind gifts from the Molecular Oncology Laboratory at the University of Chicago Medical Center.

**Preparation of adenoviruses in the 293 cells.** AdBMP2 and AdGFP were transfected in the 293 cells to amplify the viruses. Propagations of the viruses were visualized by GFP expression under a fluorescence microscope. The viral supernatant was purified in phosphate-buffered saline (PBS) by ultracentrifugation and the titration of the viruses was measured by end point dilution assays. The prepared viruses were stored at -80°C for use.

**Culture and treatment of the H9c2 cells.** The H9c2 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/high glucose (Thermo Scientific, Rochester, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) in humidified air (5% CO<sub>2</sub>) at 37°C. The cells were transfected with AdBMP2 or AdGFP at a varied multiplicity of infection (MOI). The transfection efficiency was measured by flow cytometry.

**Real-time RT-PCR.** The cultured H9c2 cells were collected 24, 48 or 72 h after transfection with AdBMP2 or AdGFP. The total RNA was extracted using a RNA extraction kit (Bioteck, Beijing, China). Single-stranded cDNA was reverse transcribed from 500-1,000 ng RNA using oligo(dT)-adaptor primers and AMV reverse transcriptase (Takara, Otsu, Japan) according to the manufacturer's instructions. The cDNA was then amplified using gene-specific primers (Takara Biotechnology, Dalian, China) and a SYBR-Green Dye kit (Tiangen, Beijing, China). The mRNA expression levels of

BMP2, GATA4, MEF2C, Tbx5, p300, GCN5 and  $\beta$ -actin were quantified by real-time RT-PCR using an IQcycler kit (Bio-Rad, Hercules, CA, USA). The annealing temperatures were 54°C for BMP2, 57°C for GATA4 and Tbx5 and 65°C for MEF2C, p300, GCN5 and  $\beta$ -actin. The gene-specific primers were designed using the Primer-3 software as follows: BMP2 forward, 5'-gacatccactccacaaacgaga-3' and reverse, 5'-gtcattccacccacatcact-3'; GATA4 forward, 5'-caactgc-cagactaccaccac-3' and reverse, 5'-ccatggagcttc atgtagagg-3'; MEF2C forward, 5'-gcgaagtgcgattgatgaaga-3' and reverse, 5'-gtggatgtcagtgctggcgta-3'; Tbx5 forward, 5'-cctgggtc-cgtaggtggaatag-3' and reverse, 5'-ctttgatgtct gtctcggtag-3'; p300 forward, 5'-agattcagaggcagcagagac-3' and reverse, 5'-gccataggaggtgggttcatac-3'; GCN5 forward, 5'-ggaaaggaga-agggaaggag-3' and reverse, 5'-gtcaatggggaa gcggataac-3';  $\beta$ -actin forward, 5'-ggagattactgccctggctccta-3' and reverse, 5'-gactcatgtactctctgtctgct-3'. The analyses of relative mRNA expression were carried out using the  $2^{-\Delta\Delta Ct}$  method as described previously (22). The values were normalized using  $\beta$ -actin as an endogenous housekeeping control gene.

**Western blot analysis.** The cultured H9c2 cells were collected 24 h after transfection with AdBMP2 or AdGFP. Nuclear proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction kit (KeyGen, Nanjing, China) according to the manufacturer's instructions. The nuclear proteins were separated and electrophoresed on 15% Bis-Tris polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF blots were then blocked with 5% nonfat milk in PBST (PBS plus 0.05% Tween-20) for 1 h. Subsequently, the blots were incubated with rabbit monoclonal antibodies for acetylated histone H3 (Ac-H3; Millipore, Temecula, CA, USA; 1:500 dilution) or for histone H3 (Millipore, Charlottesville, VA, USA; 1:500 dilution) in PBST containing 5% nonfat milk at 4°C overnight. HRP-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used as the secondary antibodies. The immunoreactive protein bands were detected with an Enhanced Chemiluminescence Luminal reagent (KeyGen), then scanned with a chemiluminescence kit (Gene, Hong Kong, China) and analyzed with Quantity One version 4.4 software (Bio-Rad). All western blotting experiments were repeated a minimum of 3 times.

**HAT activity assay.** The nuclear proteins were extracted as mentioned previously. The HAT activities of the nuclear protein extraction were determined using a HAT Activity Colorimetric Assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. Nuclear proteins (40  $\mu$ g) were prepared for each assay in 96-well plates at a concentration of 1  $\mu$ g/ $\mu$ l, with 40  $\mu$ l water prepared in one plate for the background reading. The assay mix was then added to each well and incubated at 37°C for 4 h. The samples were read by an enzyme micro-plate reader (Thermo Scientific) at a wavelength of 440 nm. Background readings were subtracted from the reading of all the samples. The HAT activities were expressed as relative OD values.

**Chromatin immunoprecipitation (ChIP) assay.** The cultured H9c2 cells were washed with PBS 48 h after treatment with

AdBMP2 or AdGFP, then the cells were fixed with formaldehyde to cross link the proteins and DNA. ChIP experiments were performed using a ChIP assay kit (Millipore, Billerica, MA, USA). Subsequent to cross-linking, the DNA was cut into small fragments by sonication. The conditions used for the sonication to shear the DNA were 10 sec/time, with an interval of 80 sec for cooling. These steps were repeated 270 times. The protein-DNA complex was then recruited and precipitated by rabbit monoclonal antibodies for Ac-H3 (ChIP grade; Millipore). Anti-RNA polymerase was used as a positive control and normal mouse IgG was used as a negative control. The DNA from the samples was then obtained by phenol/chloroform extraction and ethanol precipitation. The promoter regions of GATA4, MEF2C and Tbx5 were assayed by real-time PCR of the total DNA using specific primers. The sequences of these primers were as follows: GATA4 forward, 5'-actgac gccgactccaaactaag-3' and reverse, 5'-gtgtccctgttctccctgtagc-3'; MEF2C forward, 5'-cttccaggttgctcttactcc-3' and reverse, 5'-gcctctctactaacaagtggtga-3'; Tbx5 forward, 5'-actgac gccgactccaaactaag-3' and reverse, 5'-gtgtccctgttctccctgtagc-3'. The annealing temperatures were 57°C for Tbx5 and 65°C for GATA4 and MEF2C. The analyses of the relative promoter precipitation levels were carried out using the  $2^{-\Delta\Delta C_t}$  method as described previously (22). The values were normalized using an input sample as the internal standard.

**Statistical analysis.** All experiments were repeated independently at least 3 times. All data are reported as mean  $\pm$  SD and statistical analyses was performed using a one-way ANOVA.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of BMP2 in the H9c2 cells.** To identify the expression of BMP2 in the H9c2 cells following transfection with AdBMP2, BMP2 mRNA levels were measured using real-time RT-PCR assays. The expression of the  $\beta$ -actin gene was used as an endogenous control. BMP2 was highly expressed in the AdBMP2-transfected cells, reaching a peak 48 h after the transfection (1,432 $\pm$ 313-fold, AdBMP2 group vs. blank group,  $P < 0.05$ ). Extremely low expression levels of BMP2 were detected in the AdGFP and blank groups (Fig. 1A).

**Expression of GATA4 and MEF2C in H9c2 cells overexpressing BMP2.** The results of real-time RT-PCR assays showed that the mRNA expression levels of GATA4 and MEF2C were significantly enhanced in H9c2 cells overexpressing BMP2. As shown in Fig. 1B and C, GATA4 and MEF2C expression levels were enhanced by BMP2 and reached a peak 48 h after AdBMP2 transfection (1.94 $\pm$ 0.23-fold for GATA4 and 4.84 $\pm$ 0.43-fold for MEF2C, AdBMP2 vs. blank group,  $P < 0.05$ ). The expression levels of GATA4 and MEF2C in the AdGFP group were not altered (AdGFP vs. blank group,  $P > 0.05$ ). In addition, there was no significant change observed in the expression of Tbx5 in the cells following AdBMP2 transfection (AdBMP2 vs. blank group,  $P > 0.05$ ; Fig. 1D).

**Increased histone H3 acetylation in the whole chromatin of H9c2 cells overexpressing BMP2.** Using western blot-

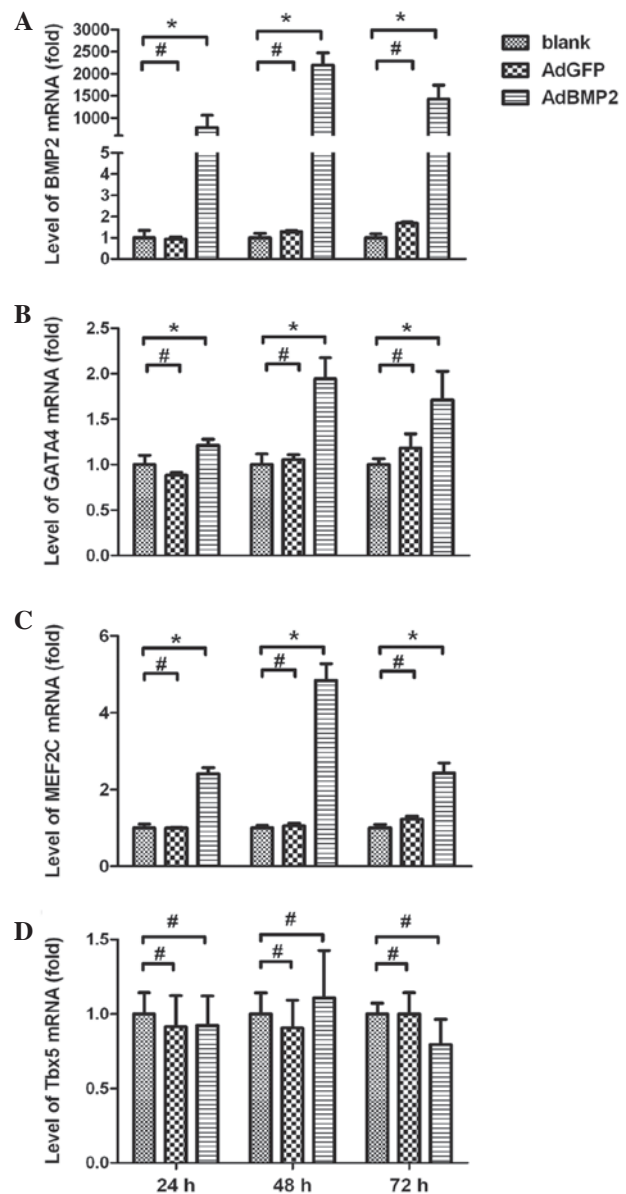


Figure 1. Expression of GATA4, MEF2C and Tbx5 in the H9c2 cells transfected with recombinant adenoviruses expressing human BMP2 (AdBMP2). The H9c2 cells were transfected with AdBMP2 or control adenoviruses expressing green fluorescent protein (AdGFP). The mRNA expression levels of (A) BMP2, (B) GATA4, (C) MEF2C and (D) Tbx5 were detected in the H9c2 cells 24, 48 or 72 h after transfection. (A) Real-time RT-PCR results showed that BMP2 was overexpressed in the AdBMP2-transfected cells compared with the blank control cells. (B) The mRNA expression levels of GATA4 were increased by BMP2 and reached a peak 48 h after AdBMP2 transfection. (C) The mRNA expression levels of MEF2C were increased by BMP2 and reached a peak 48 h after AdBMP2 transfection. (D) The mRNA expression levels of Tbx5 were not affected in the same cells following AdBMP2 transfection. \* $P < 0.05$ ; # $P > 0.05$ . BMP2, bone morphogenetic protein-2.

ting assays, the effect of BMP2 on histone H3 acetylation in H9c2 cells overexpressing BMP2 was analyzed. The ratio of Ac-H3 to H3 was determined in order to normalize the sample recovery and loading. As shown in Fig. 2, the histone H3 acetylation levels were significantly increased in H9c2 cells overexpressing BMP2 (3.07 $\pm$ 0.16-fold, AdBMP2 vs. blank group,  $P < 0.05$ ). There was no significant difference in histone H3 acetylation levels between the AdGFP and blank groups ( $P > 0.05$ ).



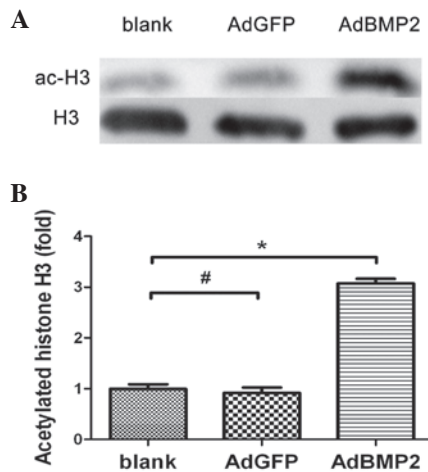


Figure 2. Effects of BMP2 on histone H3 acetylation in the whole chromatin extracted from H9c2 cells overexpressing BMP2. (A) Representative western blot. (B) Summary of the results from three independent western blotting assays indicating that the total amount of acetylated histone H3 (Ac-H3) in the whole chromatin was increased significantly in the cells 48 h after AdBMP2 transfection. \* $P < 0.05$ ; # $P > 0.05$ . BMP2, bone morphogenetic protein-2; AdBMP2, adenoviruses expressing BMP2; AdGFP, adenoviruses expressing green fluorescent protein.

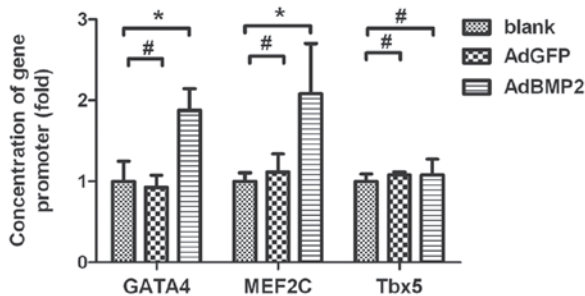


Figure 3. Effects of BMP2 on histone H3 acetylation in the promoter regions of GATA4, MEF2C and Tbx5 in H9c2 cells overexpressing BMP2. The DNA quantities of GATA4 and MEF2C immunoprecipitated by the antibody for Ac-H3 were increased significantly in the H9c2 cells 48 h after AdBMP2 transfection, indicating an increased acetylation level of histone H3 in the promoter regions of these genes. The histone H3 acetylation level in the promoter region of Tbx5 was not changed. \* $P < 0.05$ ; # $P > 0.05$ . BMP2, bone morphogenetic protein-2; AdBMP2, adenoviruses expressing BMP2; AdGFP, adenoviruses expressing green fluorescent protein.

**Increased histone H3 acetylation in the promoter regions of GATA4 and MEF2C in H9c2 cells overexpressing BMP2.** ChIP and real-time PCR assays were conducted to further investigate whether the upregulation of GATA4 and MEF2C by BMP2 was associated with changes in the histone H3 acetylation of these genes. The real-time PCR results showed that the quantities of promoter DNA for GATA4 and MEF2C were increased in H9c2 cells overexpressing BMP2 ( $1.88 \pm 0.27$ -fold for GATA4,  $2.08 \pm 0.62$ -fold for MEF2C, AdBMP2 vs. blank group,  $P < 0.05$ ). There was no significant difference in these values between the AdGFP and blank groups ( $P > 0.05$ ; Fig. 3). Accordingly, the ChIP data indicated that the histone H3 acetylation levels in the promoter regions of GATA4 and MEF2C were significantly increased in H9c2 cells overexpressing BMP2. However, the histone H3 acetylation in the promoter region of Tbx5 was not significantly changed in the same

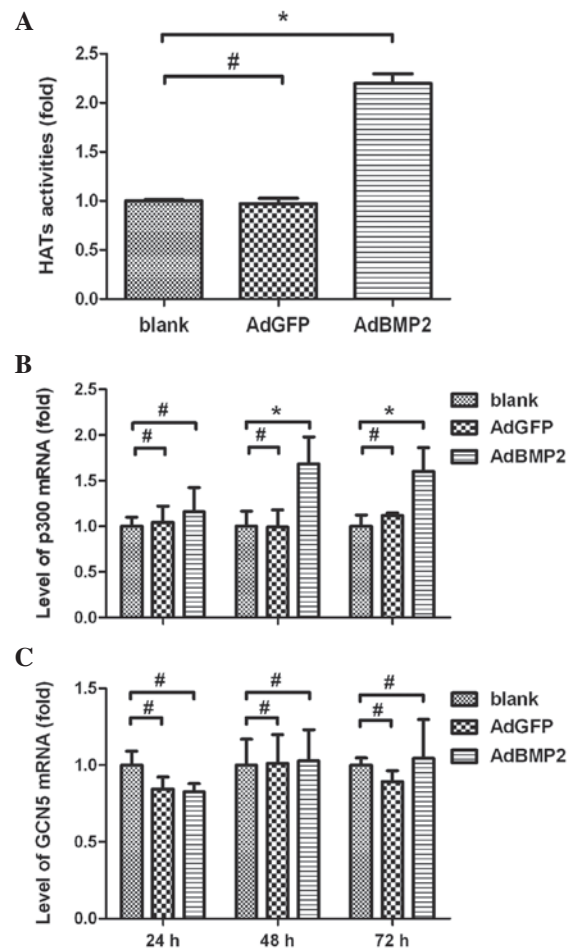


Figure 4. Effects of BMP2 on the HAT activities and the expression of HAT subtypes p300 and GCN5 in H9c2 cells overexpressing BMP2. (A) The HAT activities were increased significantly in H9c2 cells 48 h after AdBMP2 transfection. (B) Real-time RT-PCR assay showed that the mRNA expression levels of p300 were upregulated in the cells after AdBMP2 transfection. (C) No alterations on the expression of GCN5 mRNA were observed in the same cells after AdBMP2 transfection. \* $P < 0.05$ ; # $P > 0.05$ . HAT, histone acetylase; BMP2, bone morphogenetic protein-2; AdBMP2, adenoviruses expressing BMP2; AdGFP, adenoviruses expressing green fluorescent protein.

cells overexpressing BMP2 ( $P > 0.05$ ). These data indicate that increased expression of GATA4 and MEF2C are associated with increased histone H3 acetylation in the promoter regions of these genes in H9c2 cells overexpressing BMP2.

**Enhanced HAT activities and increased expression of HAT subtype p300 in H9c2 cells overexpressing BMP2.** To further investigate whether the BMP2-induced histone H3 hyperacetylation was associated with HATs, experiments were performed using colorimetric assays to measure the HAT activities and to determine the expression levels of the HAT p300 subtype in H9c2 cells overexpressing BMP2. The results showed that HAT activities were significantly increased in H9c2 cells overexpressing BMP2 ( $2.20 \pm 0.17$ -fold, AdBMP2 vs. blank group,  $P < 0.05$ ). There were no significant differences in the HAT activities between the AdGFP and blank groups ( $P > 0.05$ ; Fig. 4A). The real-time RT-PCR data showed that the expression levels of p300 were significantly increased and reached a peak in the H9c2 cells 48 h after AdBMP2 transfection ( $1.68 \pm 0.30$ -fold, AdBMP2 group vs. blank

group,  $P < 0.05$ ). There was no significant difference in the p300 expression levels between the AdGFP and blank groups ( $P > 0.05$ ; Fig. 4B). Moreover, the expression of GCN5 was not significantly altered in H9c2 cells overexpressing BMP2 ( $P > 0.05$ ; Fig. 4C).

## Discussion

GATA4, MEF2C and Tbx5 are the most significant transcription factors during the early stage of heart development (6). In the present study, the expression levels of GATA4 and MEF2C, but not Tbx5, were identified as significantly enhanced in H9c2 cells overexpressing BMP2. These data are in agreement with the previous reports that BMP2 is able to induce GATA4 and MEF2C expression ectopically, resulting in an ectopic cardiac mesoderm specification in chicken embryos (9). These studies also observed that BMP2 was not able to affect the expression of Tbx5 in chicken embryos (9,14). Consistent with these studies, the results of the present study confirm that the expression of Tbx5 is not altered in H9c2 cells overexpressing BMP2.

Numerous studies have reported that BMP2 is required for heart development by inducing expression in various cardiac genes. However, the underlying mechanisms are largely unknown. One previous study in bovine granulosa cells suggested that BMP4 was able to suppress the expression of StAR by inhibiting histone H3 acetylation in the promoter regions of the gene (23). Pan *et al* (24) suggested that the activation of Sox9 gene transcription by BMP2 was associated with chromatin remodeling and histone modification in primary mouse embryo fibroblasts. In the present study, western blotting assays were performed to determine whether BMP2 was able to affect the histone H3 acetylation in H9c2 cells overexpressing BMP2. First an increase in the total amount of Ac-H3 in the whole chromatin extracted from H9c2 cells overexpressing BMP2 was identified. Further analysis of histone H3 acetylation in the promoter regions of the specific cardiac genes was then conducted. The results demonstrate that BMP2 is able to enhance histone H3 acetylation levels in the promoter regions of GATA4 and MEF2C. The increase of histone H3 acetylation levels in these genes is accompanied by an increase in the expression levels of these genes, suggesting that histone H3 acetylation may be one of the molecular mechanisms by which BMP2 upregulates the expression of GATA4 and MEF2C in H9c2 cells overexpressing BMP2. By contrast, no change in Tbx5 expression or in histone H3 acetylation in the Tbx5 gene promoter was observed in the same cells overexpressing BMP2.

The status of histone acetylation is determined by the balanced actions between the HATs and HDACs. In the present study, the colorimetric assay data show that BMP2 is able to enhance the HAT activities in H9c2 cells overexpressing BMP2. However, it is unclear which HAT subtype(s) participates in the regulation of histone H3 acetylation in response to BMP2 in the H9c2 cells. In our previous studies, p300 was identified with a higher expression level than other HAT subtype in crescent-shaped cardiogenic plates (25), and inhibition of p300 HAT activity resulted in reduced histone acetylation and decreased expression of GATA4 and MEF2C in mouse cardiac myocytes (26). Other studies also indicated a critical role for p300 in cardiac gene expression and heart development (27). In

the present study, the data indicate that the expression level of p300 was significantly increased in H9c2 cells overexpressing BMP2, while another HAT subtype, GCN5, had no significant changes in the same cells overexpressing BMP2. This suggests that p300 is more important than GCN5, in BMP2-induced histone H3 hyperacetylation in the H9c2 cells.

In conclusion, the data demonstrate that BMP2 upregulates histone H3 acetylation levels in the promoter regions of specific cardiac genes, GATA4 and MEF2C. BMP2 also increases HAT activities and the expression of the HAT p300 subtype. The upregulatory effects of BMP2 on GATA4 and MEF2C are due, at least in part, to the increased acetylation levels of histone H3 in the promoter regions of these genes, which is associated with increased HAT activities and the expression of the HAT p300 subtype in the H9c2 cells.

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