Bone morphogenetic protein 4 promotes the differentiation of Tbx18-positive epicardial progenitor cells to pacemaker-like cells

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Abstract. Clarifying the mechanisms via which pacemaker-like cells are generated is critical for identifying novel targets for arrhythmia-associated disorders and constructing pacemakers with the ability to adapt to physiological requirements. T-box 18 (Tbx18)$^+$ epicardial progenitor cells (EPCs) have the potential to differentiate into pacemaker cells. Although bone morphogenetic protein 4 (Bmp4) is likely to contribute, its role and regulatory mechanisms in the differentiation of Tbx18$^+$ EPCs into pacemaker-like cells have remained to be fully elucidated. In the present study, the association between Bmp4, GATA binding protein 4 (Gata4) and hyperpolarization-activated cyclic nucleotide gated potassium channel 4 (Hcn4) to regulate NK2 homeobox 5 (Nkx2.5), which is known to be required for the differentiation of Tbx18$^+$ EPCs into pacemaker-like cells, was assessed. Tbx18$^+$ EPCs were isolated from Tbx18:Cre/Rosa26R$^{\text{enhanced yellow fluorescence protein (EYFP)}}$ murine embryos at embryonic day 11.5 and divided into the following four treatment groups: Control, Bmp4, Bmp4+LDN193189 (a Bmp inhibitor) and LDN193189. *In vitro* Bmp4 promoted the expression of Hcn4 in Tbx18$^+$ EPCs via lineage tracing of Tbx18:Cre/Rosa26R^{EYFP} mice, which was likely due to upregulation of Gata4 expression. Gata4 knockdown experiments were then performed using the following five treatment groups: Control, control small interfering RNA (siRNA), Bmp4, Bmp4+siRNA targeting Gata4 (siGata4) and siGata4 group. Knockdown of Gata4 caused a downregulation of Hcn4 and an upregulation of Nkx2.5, but had no effect on Bmp4 expression. In conclusion, it was indicated that in Tbx18$^+$ EPCs, the expression of Nkx2.5 was regulated by Bmp4 via Gata4. Taken together, these results provide important information on regulatory networks of pacemaker cell differentiation and may serve as a basis for further studies.

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Introduction

The sinoatrial node (SAN), located between the superior vena cava and right atrium, is a natural cardiac pacemaker. Understanding the mechanisms of pacemaker cell development during embryogenesis is important for treating SAN defects and constructing pacemakers that capable to adapt to physiological requirements. Previous studies have confirmed that T-box 18 (Tbx18) has key roles in heart development, particularly in the formation of the SAN (1-4). Multiple studies investigating SAN head dysplasia in Tbx18 knock-out models indicated that Tbx18 functions in SAN structure formation (2,3,5). Multipotent epicardial progenitor cells (EPCs) originate from the proepicardial organ, a temporary structure outside the embryonic heart that expresses transcription factors including Tbx18, Wnt1 and transcription factor 21 (2,6-8). Cells migrate from the pro-epicardial organ to cover the surface of the embryonic heart and form the epicardium. Studies have confirmed that most of the epicardial cells express the Tbx18 transcription factor (9). Tbx18-positive (Tbx18$^+$) pro-epicardium develops into the SAN as a process of epicardium formation (1,3,10,11). Therefore, Tbx18$^+$ EPCs may be among the best candidates to investigate the mechanisms of SAN differentiation and generate biological pacemakers. However, the differentiation process of Tbx18$^+$ EPCs into pacemaker cells has not been elucidated.

Bone morphogenetic protein (Bmp), a member of the transforming growth factor β superfamily, regulates various processes during embryonic development. Disruption of Bmp4 (homozygous mutant embryos following homologous recombination in embryonic stem cells) in mice is lethal to embryos in the early gastrulation period [embryonic day (E)6.5-E9.5] (12). In addition to its roles in the development of bone tissues, Bmp4 has a key role in embryonic heart development. Changes in Bmp4 localization affect heart patterning and looping (13). Furthermore, loss of Bmp4 expression may lead to the development of abnormal cardiac structures (14). In addition to its influence on the development of cardiac structures, Bmp4 promotes fibroblast reprogramming to cardiomyocytes that have spontaneous pacemaker activity in embryonic mice (15). Furthermore, Bmp4 is a direct target of Shox2 (short stature homeobox 2), which has a key role in SAN development, and the expression patterns of Bmp4 and Shox2 overlap in the embryonic SAN (16). Bmp4 has an important role in the differentiation of pacemaker cells;

however, the role of Bmp4 in the differentiation of Tbx18+ EPCs to pacemaker cells has remained to be explored. The aim of the present study was to determine whether Bmp4 regulates the differentiation of Tbx18+ EPCs into pacemaker cells using Tbx18:Cre/Rosa26R$^{\text{enhanced yellow fluorescence protein (EYFP)}}$ lineage tracing models *in vitro*.

Materials and methods

Transgenic mice and primary culture of Tbx18+ EPCs. All animal experiments were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (Chongqing, China) and were in compliance with the 'Legislation for the Protection of Animals used for Scientific Purposes' of the P.R. China. Tbx18:Cre knock-in transgenic mice (Evans Laboratory; University of California, San Diego, CA, USA) and Rosa26R^{EYFP} (Jackson Laboratory; Bar Harbor, ME, USA) mice were bred on a C57BL/6 background obtained from the animal center of Chongqing Medical University. E11.5 double-transgenic embryos were isolated from Tbx18:Cre female mice that were mated with Rosa26R^{EYFP} male mice. Atria and outflow tract tissues were removed, and EPCs were allowed to grow out from the retained ventricles. After the ventricles were removed, EPCs were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The procedure used to obtain the EPCs was in accordance with previous studies (9,17). Tbx18+ EPCs were separated into the following four treatment groups: Control, Bmp4 (60 ng/ml; cat. no. P5958; Abnova, Taipei, Taiwan), Bmp4+LDN193189 [final concentration of Bmp4, 60 ng/ml and LDN193189 (final concentration, 0.5 μmol/l; cat. no. HY-12071; MedChem Express, Monmouth Junction, NJ, USA)] and LDN193189 (0.5 μmol/l). The Bmp4+LDN193189 group was pre-treated with LDN193189 for 30 min prior to simultaneous treatment with Bmp4 and LDN193189. The culture medium, which contained the drugs used to treat each group, was changed every two days over a total of 6 days.

Knockdown of Gata4 in EPCs. Knockdown experiments were performed using EPCs from wild-type C57BL/6 mice since almost all EPCs were Tbx18+ cells, as demonstrated by the current study and a previous study (17). Tbx18+, which was conjugated to YFP in the mice, was identified in EPCs using immunofluorescence. Cells were transfected with Gata4-specific small interfering (si)RNA (siGata4; cat. no. MSS247225) or control siRNA (siControl; cat. no. 12935-300) with Lipofectamine RNAiMAX (cat. no. 13778-150; all Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols, and cultured for 72 h without any additional media changes. Cells were divided into the following five treatment groups: Control, siControl, Bmp4, Bmp4+siGata4 and siGata4.

Immunofluorescence. The culture medium was removed and cells, which were grown on cover slips without coated, were fixed with 4% paraformaldehyde at room temperature for 15 min, followed by permeabilization with 0.25% Triton X-100 in PBS (0.01 mol/l) at 37˚C for 10 min. Cells were blocked with 10% goat serum (Boster Biological Technology, Pleasanton, CA, USA) at 37˚C for 10 min, and then incubated with the following primary antibodies at 4˚C overnight: Hyperpolarization-activated cyclic nucleotide gated potassium channel 4 (Hcn4; 1:200 dilution; cat. no. ab69054; Abcam, Cambridge, UK), Bmp4 (1:200 dilution; cat. no. MAB1049; EMD Millipore, Billerica, MA, USA), Gata4 (1:200 dilution; ab134057) and NK2 homeobox 5 (Nkx2.5; 1:150 dilution; cat. no. ab91196; both Abcam). The cells were then incubated with cyanine 3-conjugated goat anti-rabbit immunoglobulin G (CWBIO, Beijing, China) at 37˚C for 45 min, followed by DAPI at room temperature for 10 min. Subsequently, the cells were subjected to confocal microscopy imaging (original magnification, x400). Imaging conditions for each antibody were kept consistent across all samples.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were collected and lysed with TRIzol (Takara Bio Inc., Otsu, Japan) to extract total RNA, which was reverse-transcribed to complementary DNA using a PrimeScript Reverse Transcriptase reagent kit (cat. no. RR047A; Takara Bio Inc.) according to the manufacturer's protocols. qPCR was performed with SYBR premix Ex Taq (cat. no. RR820A; Takara Bio Inc.) on a C1000 thermal cycler (BioRad Laboratories, Hercules, CA, USA) using the following thermocycling conditions: 95˚C for 35 sec, and 40 cycles of 95˚C for 35 sec, 60˚C for 30 sec and 72˚C for 30 sec. qPCR for Nkx2.5 was performed under similar conditions with 46 cycles due to its low mRNA expression levels. GAPDH was used as an internal reference for each gene. Primers were provided by Sangon Biotech Co. Ltd. (Shanghai, China) and their sequences are listed in Table S1. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to GAPDH (18).

Statistical analysis. All experiments were repeated three times. Values are expressed as the mean ± standard deviation. The data were analyzed using SPSS (version 20.0; IBM Corp., Armonk, NY, USA), and one-way analysis of variance followed by a Tukey's test was applied for comparison between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Bmp4 promotes the differentiation of Tbx18+ EPCs to pacemaker-like cells. YFP expression in the EPCs, via which Tbx18 expression can be identified, was evident during immunofluorescence under confocal microscope (Figs. 1 and 2). Tbx18+ EPCs were treated with 60 ng/ml Bmp4 for 3 or 6 days. The expression of Hcn4, a marker of pacemaker cells, was upregulated in Bmp4-treated Tbx18+ EPCs that were isolated from Tbx18:Cre/Rosa26R^{EYFP} mice and confirmed by immunofluorescence analysis (Fig. 1A). The level of Hcn4 expression increased with longer durations of Bmp4 treatment (Fig. 1A-C). The effects of Bmp4 treatment were significantly blocked by LDN193189 treatment at days 3 and 6, as demonstrated by RT-qPCR analysis (both $P<0.05$; Fig. 1B and C). In addition, it was indicated that the expression levels of Hcn4 in the LDN193189-treated group were significantly lower compared with those in the control group ($P<0.05$). The mRNA levels of connexin45, a gap

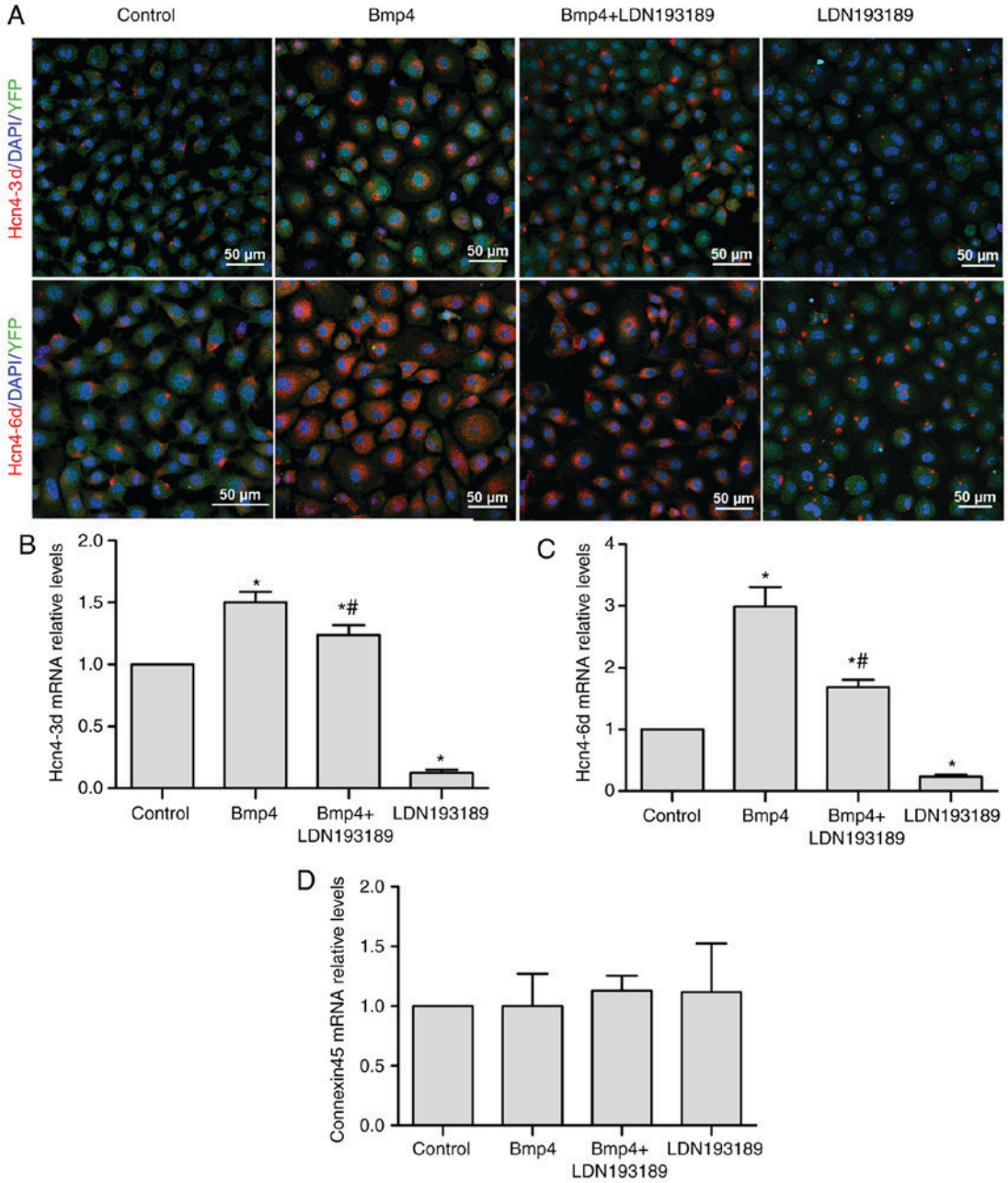

Figure 1. Bmp4 promotes the expression of pacemaker-like markers in Tbx18$^+$ EPCs. Tbx18$^+$ EPCs were treated with Bmp4, Bmp4+LDN193189 or LDN193189 for 3 days (upper panel) or 6 days (lower panel) and compared to the control group. (A) Representative immunofluorescence microscopy images of EPCs from the four groups with staining for Hcn4 (red). The nuclei were counterstained with DAPI (blue) and the YFP expressed by the cells is apparent (scale bar, 50 μm). Reverse transcription-quantitative polymerase chain reaction analysis. (B and C) the mRNA levels of Hcn4 at (B) 3 days and (C) 6 days in the four groups. The expression of Hcn4 in the Bmp4 group was higher than that in the control group, and the effect was partially blocked by the Bmp inhibitor LDN193189. (D) The mRNA levels of connexin45 were not significantly different at 6 days between the 4 groups. Values are expressed as the mean ± standard deviation. *P<0.05 vs. the control group; #P<0.05 vs. the Bmp4 group. EPCs, epicardial progenitor cells; Bmp, bone morphogenetic protein; YFP, yellow fluorescence protein; Hcn4, hyperpolarization-activated cyclic nucleotide gated potassium channel 4; Tbx18, T-box 18.

junction protein that is expressed mainly in the SAN, did not differ between the groups (Fig. 1D).

Gata4 is a potential downstream target of Bmp4. Shox2 and Tbx3 are key regulatory factors that mediate the patterning and pacemaker function of the SAN (3,19-24). Gata4, a transcription factor critical in the development of the epicardium, may interact with Bmp4 in specific signaling pathways (25). By contrast, Nkx2.5, a transcription factor that is not abundantly expressed in the SAN, may prevent SAN development (3,26,27). In the present study, the expression of these transcription factors was determined by analyzing the downstream signaling molecules of Bmp4 in Tbx18$^+$ EPCs. Gata4 was expressed in >99% of Tbx18$^+$ EPCs and was mainly localized in the nucleus (Fig. 2A). The addition of Bmp4 for 6 days upregulated nuclear expression of Gata4 protein in Tbx18$^+$ EPCs (Fig. 2A) and significantly increased Gata4 mRNA levels compared with the control group ($P<0.05$; Fig. 2B); this upregulation was partially blocked by the co-administration of Bmp4 and LDN193189 ($P<0.05$;

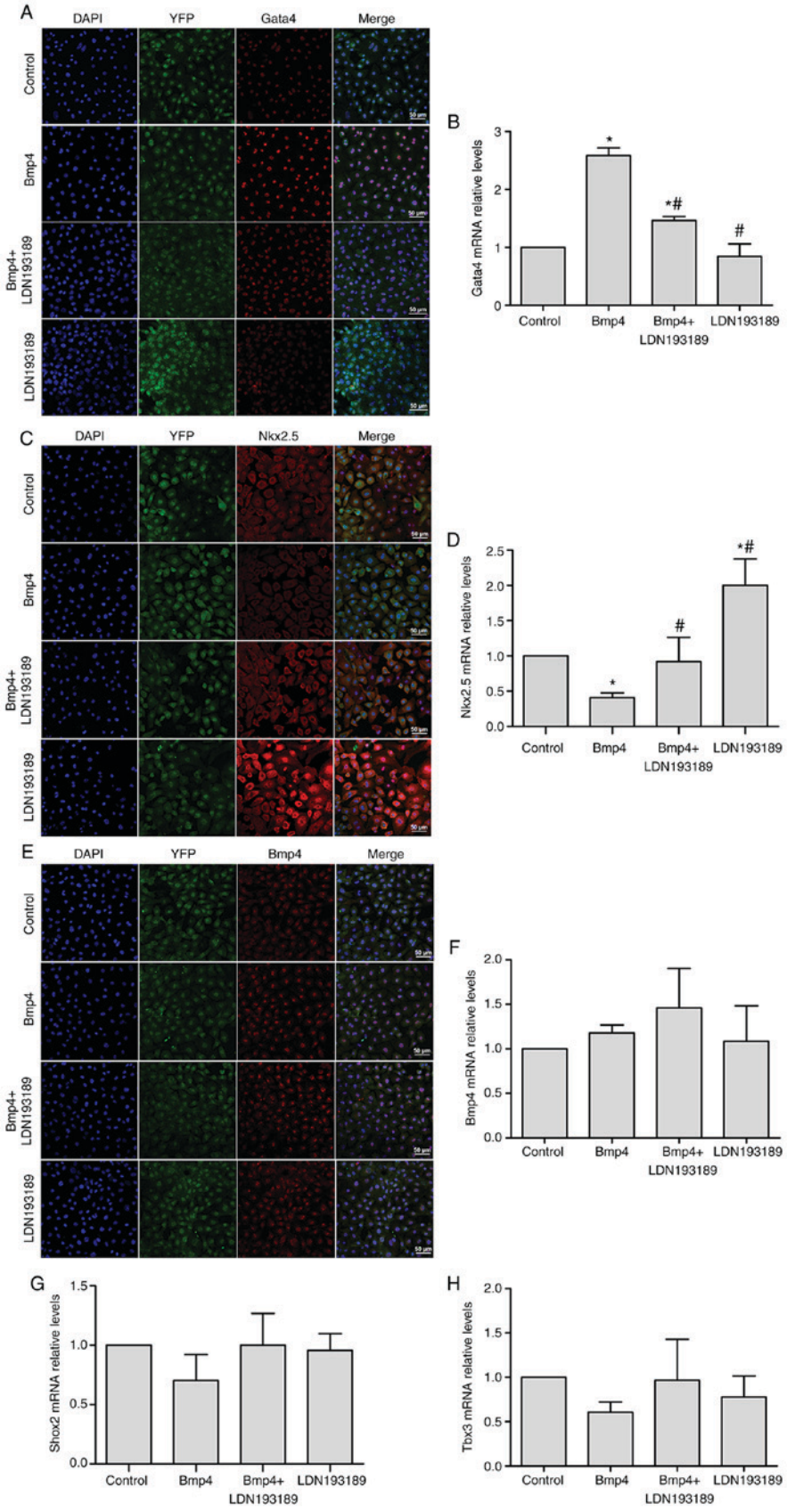

Figure 2. Gata4 is a potential downstream target of Bmp4. Bmp4 promotes the expression of Gata4 in Tbx18+ EPCs, as assessed by (A) immunofluorescent staining and (B) RT-qPCR. Inhibition of Bmp4 caused an upregulation of Nkx2.5 expression, as assessed by (C) immunofluorescent staining and (D) RT-qPCR. Treatment with exogenous Bmp4 had no significant effect on the expression of Bmp4, as indicated by (E) immunofluorescent staining and (F) RT-qPCR, and did not significantly affect (G) Shox2 and (H) Tbx3 expression in Tbx18+ EPCs. Representative immunofluorescence microscopy images are presented with Gata4 or Nkx2.5 displaying in red; the nuclei were counterstained with DAPI (blue) and the YFP expressed by the cells is apparent (scale bar, 50 μm). Values are expressed as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. the Bmp4 group. EPCs, epicardial progenitor cells; Bmp, bone morphogenetic protein; YFP, yellow fluorescence protein; Hcn4, hyperpolarization-activated cyclic nucleotide gated potassium channel 4; Tbx18, T-box 18; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Gata4, GATA binding protein 4; Nkx2.5, NK2 homeobox 5; Shox2, short stature homeobox 2.

Fig. 2A and B). The addition of the Bmp4 inhibitor caused an upregulation of Nkx2.5 expression (P<0.05; Fig. 2C and D), particularly in the cytoplasm (Fig. 2C). Furthermore, immunofluorescence staining demonstrated that Bmp4 is expressed in the nuclei of Tbx18$^+$ EPCs and that treatment with exogenous Bmp4 did not change the intracellular Bmp4 protein localization (Fig. 2E) and did not significantly affect the mRNA levels (Fig. 2F). In addition, Bmp4 had no significant effect on the expression of Shox2 or Tbx3, as indicated by RT-qPCR analysis (Fig. 2G and H). Overall, these results indicated that Bmp4 may promote the differentiation of Tbx18$^+$ EPCs to pacemaker-like cells via upregulation of Gata4 and downregulation of Nkx2.5.

Knockdown of Gata4 inhibits the differentiation of Tbx18$^+$ EPCs into pacemaker-like cells. To determine whether Gata4 has a role in the Bmp4-dependent differentiation of Tbx18$^+$ EPCs to pacemaker-like cells, a knockdown experiment with Gata4 was performed. The effectiveness of Gata4 knockdown was assessed by immunofluorescence and RT-qPCR. The results indicated that the protein and mRNA expression of Gata4 was significantly decreased at 72 h after Gata4-siRNA transfection compared with the control group (P<0.05; Fig. 3A and B, respectively). No difference in Gata4 expression was identified between the control group and the negative control group (Fig. 3A and B), indicating that knockdown of Gata4 was caused by Gata4-siRNA and no off-target effects. The expression of Hcn4, Nkx2.5 and Bmp4 after knockdown of Gata4 was then assessed. Not only did knockdown of Gata4 significantly downregulate Hcn4 mRNA expression compared with the control group (P<0.05; Fig. 3C and D), but Nkx2.5 mRNA expression was significantly and Nkx2.5protein expression was markedly upregulated (P<0.05; Fig. 3E and F). However, there was no difference in Bmp4 expression between the groups (Fig. 3G and H). The expression of Bmp4 did not differ in the Bmp4 and Bmp4+siGATA4 groups (Fig. 3G and H).

Discussion

In the present study, a primary culture of Tbx18$^+$ EPCs isolated from Tbx18:Cre/Rosa26R^{EYFP} mice was established. YFP fluorescence in the cells of Tbx18:Cre/Rosa26R^{EYFP} mice was used to identify the cells expressing Tbx18. It was indicated that Bmp4 promotes the differentiation of Tbx18$^+$ EPCs into pacemaker-like cells *in vitro*, and that Gata4 is a downstream target of Bmp4.

In the present study, ventricular EPCs were less difficult to use than atrial EPCs, particularly from the right atrium. Hcn4 is a specific marker of SAN pacemaker cells, and a majority of Tbx18$^+$ EPCs express Hcn4 protein. The ion channels formed by Hcn4 channel proteins are required to generate the current of pacemaker cells, which has a critical role in spontaneous depolarization. Knockout of Hcn4 in mice results in embryonic death (28). Tbx18$^+$ EPCs have the potential to differentiate into pacemaker cells. It has been indicated that exogenous Bmp4 promotes the mRNA and protein expression of Hcn4, suggesting that Bmp4 is a critical factor in the differentiation of Tbx18$^+$ EPCs into pacemaker-like cells. Indeed, extended treatment with Bmp4 amplified the effects on the expression of downstream targets. LDN193189, an effective Bmp4 inhibitor, inhibits BMP type I receptor kinases and subsequently the downstream signal effectors (29,30). Immunofluorescence and RT-qPCR analyses revealed that the expression levels of Hcn4 were lower in the LDN193189-treated group than in the control group. As Bmp4 was expressed in Tbx18$^+$ EPCs, it was hypothesized that Bmp4 generated by Tbx18$^+$ EPCs had an autocrine and a paracrine effect that may be partially blocked by exogenous LDN193189. Furthermore, Bmp4 treatment had no effect on the expression of Bmp4 in Tbx18$^+$ EPCs, suggesting that Bmp4 exerts its functions via a signaling mechanism through membrane receptors. This is consistent with the classical mechanism of Bmp4 (31) and with the notion that Bmp4 does not stimulate its own expression. A previous study using mouse embryonic stem cells (embryoid bodies) performed by Hashem *et al* (23) demonstrated that disruption of Shox2 downregulated Bmp4 and Hcn4, while addition of Bmp4 partially rescued this effect. This is consistent with a previous study indicating that Bmp4 directly affects the expression of Hcn4 in the development of the dorsal mesenchymal protrusions (24). Taken together, these results are consistent with those of the present study, indicating that Bmp4 is an upstream regulator of Hcn4 in Tbx18$^+$ EPCs. Hcn4 and Connexin45 are specific markers of pacemaker cells. Connexin45, but not connexin 40 or connexin 43, is expressed in pacemaker cells (32-34). In the present study, no changes in connexin45 mRNA expression were observed after Bmp4 treatment, indicating that the cells may have differentiated into pacemaker-like cells lacking this feature. However, Hcn4 was affected.

Tbx3 is expressed in the embryonic SAN (20). Loss of Tbx3 in the SAN leads to expression of mature myocardium-specific genes, while abnormal expression of Tbx3 upregulates the expression of Hcn4, forming a pacemaker in the atria (21,35,36). However, Tbx3 is not required for the formation of the SAN structure (3). In addition, the expression of Shox2 is restricted to the sinoatrial node and the venous valves. Shox2-deficient embryos have markedly decreased SAN, dysfunctional cardiac pacemaker activity and reduced Hcn4 expression (26,37,38). The present results indicated that Bmp4 promotes Hcn4 expression via upregulation of Gata4, while transcription of Shox2 and Tbx3 was not affected by Bmp4. However, the present study also suggested that Tbx18$^+$ EPCs do not abundantly express the Nkx2.5 transcription factor, which is consistent with the results of other studies (3,39). Taken together, it is indicated that Nkx2.5 inhibits SAN differentiation, and its expression is regulated by Bmp4 and Gata4.

In the present study, the mRNA and protein expression of Gata4 was effectively silenced by siGata4. There was no significant difference in the mRNA expression of Gata4 between the Bmp4+siGata4 group and the siGata4 group even though Bmp4 upregulated Gata4, which may be attributed to transcriptional gene silencing of Gata4. However, Hcn4 expression levels were higher in the Bmp4+siGata4-treated group compared with those in the siGata4-treated group, indicating that there may be other transcription factors in the same regulatory network compensating for Hcn4 expression. In addition, almost all of the EPCs isolated from the Tbx18:Cre/Rosa26R^{EYFP} mice were Tbx18$^+$ according to the immunofluorescence analysis. These results indicated the EPCs used in the Gata4 silencing experiment were Tbx18 positive as well. The expression of Gata4 and Nkx2.5 were

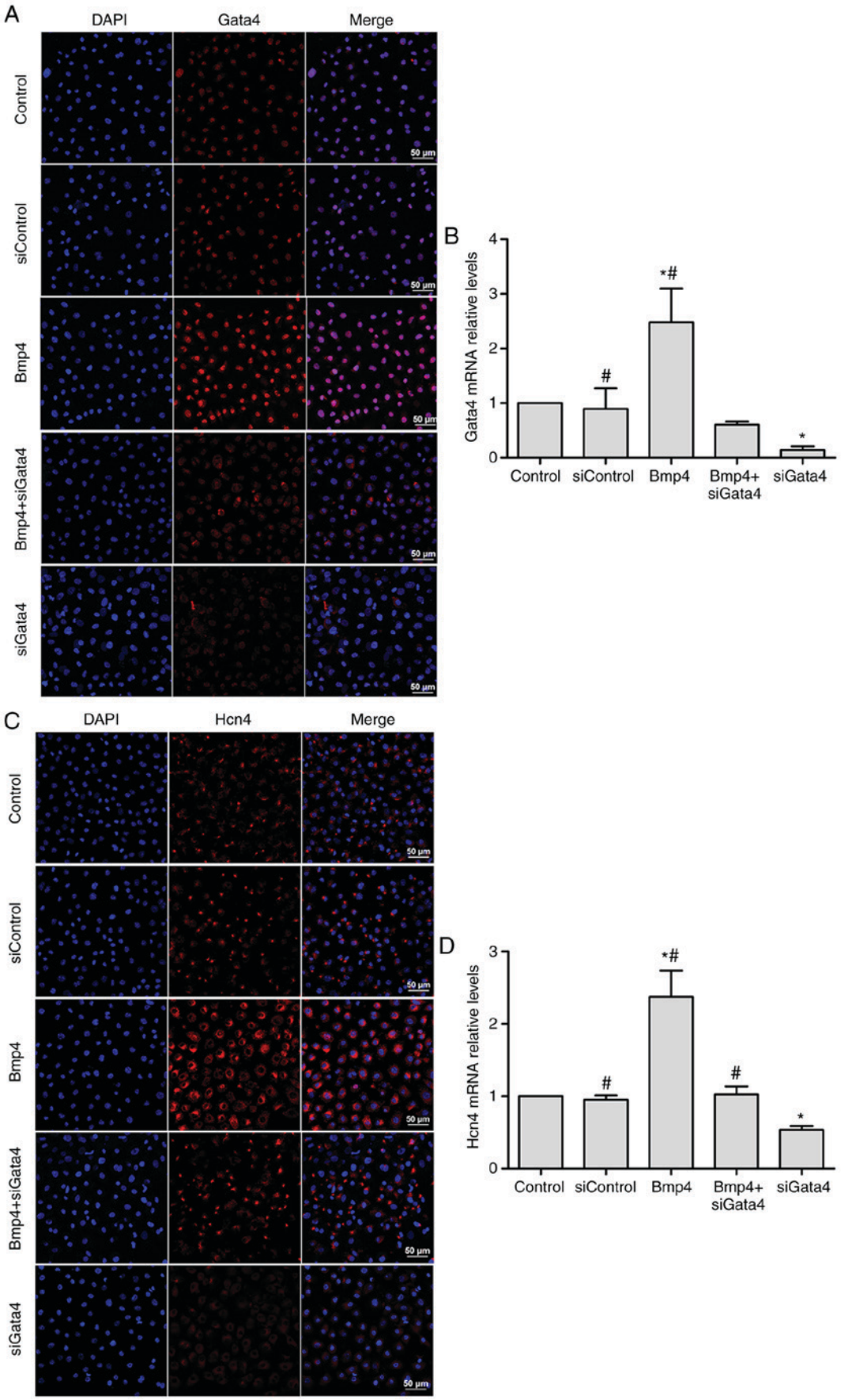

Figure 3. Effects of Gata4 knockdown in Tbx18+ epicardial progenitor cells. (A and B) Confirmation of the efficacy of Gata4 knockdown by siGata4. Knockdown of Gata4 (C and D) decreased Hcn4 expression. Representative immunofluorescence microscopy images in (A and C) are presented with Gata4 and Hcn4, respectively. Gata4 and Hcn4 expression is displayed in red; the nuclei were counterstained with DAPI (blue; scale bar, 50 μm). The mRNA expression shown in (B and D) was assessed by reverse transcription-quantitative polymerase chain reaction. Values are expressed as the mean ± standard deviation. *P<0.05 vs. the control group; #P<0.05 vs. the siGata4 group.

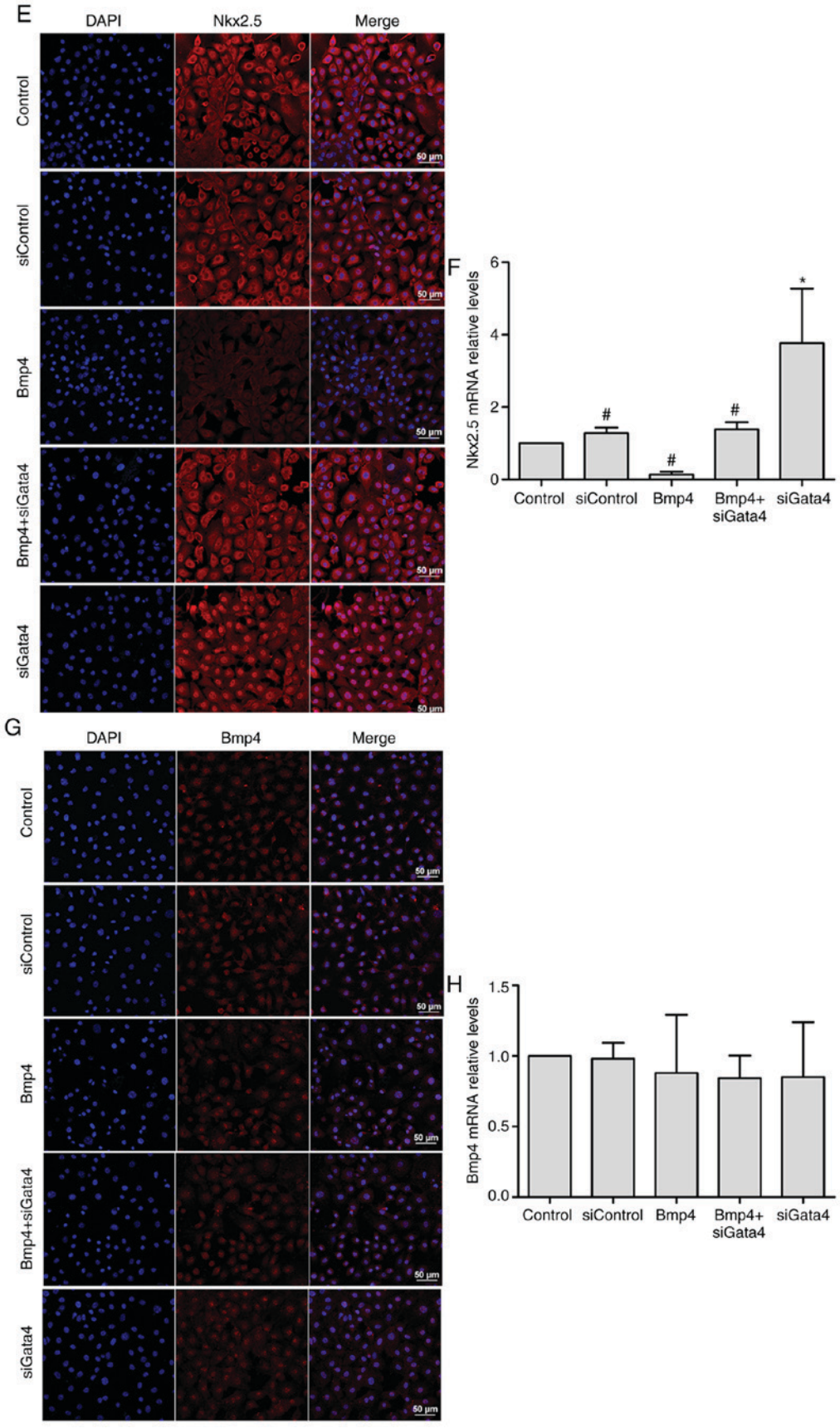

Figure 3. Continued. Effects of Gata4 knockdown in Tbx18^{+} epicardial progenitor cells. Knockdown of Gata4 (E and F) increased Nkx2.5 expression, but (G and H) did not affect Bmp4 expression levels. Representative immunofluorescence microscopy images in (E and G) are presented with Nkx2.5 and Bmp4, respectively. Nkx2.5 and Bmp4 expression is displayed in red; the nuclei were counterstained with DAPI (blue; scale bar, 50 μm). The mRNA expression shown in (F and H) was assessed by reverse transcription-quantitative polymerase chain reaction. Values are expressed as the mean ± standard deviation. *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the siGata4 group. Gata4, GATA binding protein 4; siGata4, small interfering RNA targeting Gata4; Bmp, bone morphogenetic protein; YFP, yellow fluorescence protein; Hcn4, hyperpolarization-activated cyclic nucleotide gated potassium channel 4; Tbx18, T-box 18; Nkx2.5, NK2 homeobox 5.

affected by Bmp4, while Gata4 affected the expression of Nkx2.5. Inhibition of Nkx2.5 is essential for the differentiation of EPCs into pacemaker cells (26), and Nkx2.5 expression changes their fate to form the SAN as a vital part of the working myocardium (38,40). It may therefore be speculated that high levels of Nkx2.5 in EPCs after knockdown of Gata4 downregulate Hcn4 expression to promote their differentiation into atrial myocytes. In addition, the low mRNA expression level of Nkx2.5 according to qPCR results in the current study may mean that these results are dubious. **In conclusion, the present study explored the association** between Bmp4, Gata4 and Hcn4 in Tbx18+ EPCs and revealed that the expression of Nkx2.5 is regulated by Bmp4 and Gata4, providing important information for further studies.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

QS and LW contributed to the conception of the study and were major contributors in writing the manuscript. JD and XJ made substantial contributions to the analysis and interpretation of data and critical reading of the manuscript. YY, SD and ZH contributed to the acquisition of data and helped perform the analysis, including constructive discussions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments in the present study were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University and were in compliance with the 'Legislation for the Protection of Animals used for Scientific Purposes' of the P.R. China.

Patients' consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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