Akt1 signaling coordinates BMP signaling and β-catenin activity to regulate second heart field progenitor development

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ABSTRACT

Second heart field (SHF) progenitors exhibit continued proliferation and delayed differentiation, which are modulated by FGF4/8/10, BMP and canonical Wnt/β-catenin signaling. PTEN-Akt signaling regulates the stem cell/progenitor cell homeostasis in several systems, such as hematopoietic stem cells, intestinal stem cells and neural progenitor cells. To address whether PTEN-Akt signaling is involved in regulating cardiac progenitors, we deleted Pten in SHF progenitors. Deletion of Pten caused SHF expansion and increased the size of the SHF derivatives, the right ventricle and the outflow tract. Cell proliferation of cardiac progenitors was enhanced, whereas cardiac differentiation was unaffected by Pten deletion. Removal of Akt1 rescued the phenotype and early lethality of Pten deletion mice, suggesting that Akt1 was the key downstream target that was negatively regulated by PTEN in cardiac progenitors. Furthermore, we found that inhibition of FOXO by Akt1 suppressed the expression of the gene encoding the BMP ligand (BMP7), leading to dampened BMP signaling in the hearts of Pten deletion mice. Cardiac activation of Akt also increased the Ser552 phosphorylation of β-catenin, thus enhancing its activity. Reducing β-catenin levels could partially rescue heart defects of Pten deletion mice. We conclude that Akt signaling regulates the cell proliferation of SHF progenitors through coordination of BMP signaling and β-catenin activity.

KEY WORDS: Second heart field, Akt, BMP, β-catenin, Mouse

INTRODUCTION

Lineage tracing and retrospective clonal analysis have identified two populations of cardiac progenitors during early mouse heart development (Kelly et al., 2001; Vincent and Buckingham, 2010; Watanabe et al., 2012). These two pools of cardiac progenitors are localized in the first heart field (FHF, or primary heart field) and the second heart field (SHF, or anterior heart field) (Brunceau, 2008; Harvey, 2002; Olson, 2006). Although the FHF contributes mainly to the left ventricle (LV), the SHF develops into the right ventricle (RV), the inflow tract and the outflow tract (OFT) (Black, 2007).

Starting at embryonic day (E) 8.5, the migration of SHF progenitors from the splanchnic mesoderm (SM) and pharyngeal mesoderm (PM) into the linear heart tube is essential for heart development in mice (Watanabe et al., 2012). Genetic studies in mice have revealed that disruption of SHF formation and migration severely impaired heart development. For instance, each deletion of Is11, Tbx5, Mef2c and Nkx2.5 (Nkx2-5 – Mouse Genome Informatics) affects the SHF development, resulting in developmental heart defects, with a single ventricle (the left ventricle) and the absence of SHF derivatives, including the RV and the OFT, being observed (Dodou et al., 2004; Hu et al., 2004; Lin et al., 1997; Prall et al., 2007; Xu et al., 2004).

SHF progenitors exhibit continued proliferation and a delay in differentiation (Srivastava, 2006; Watanabe et al., 2012). Fgf8 and Fgf10, the first molecular marker of the murine SHF, are the two important regulators that promote SHF proliferation (Ilagan et al., 2006; Kelly et al., 2001; Park et al., 2006; Watanabe et al., 2010, 2012). Canonical Wnt/β-catenin signaling also drives SHF progenitor cell proliferation (Klaus and Birchmeier, 2009; Klaus et al., 2007; Kwon et al., 2007). Bone morphogenetic protein (BMP) signaling is required to induce the SHF formation and to subsequently inhibit cardiac cell proliferation (Klaus et al., 2007; McCulley et al., 2008; Prall et al., 2007).

PTEN-Akt signaling regulates the stem cell/progenitor cell homeostasis (Cully et al., 2006; Oudit and Penninger, 2009; Sussman et al., 2011; Walsh, 2006). In several stem/progenitor cell systems, such as hematopoietic stem cells, intestinal stem cells (ISCs) and neural progenitor cells, deletion of Pten causes increased cell proliferation through Akt activation (Groszer et al., 2001; He et al., 2007; Li and Clevers, 2010).

To determine whether PTEN-Akt signaling is involved in the SHF regulation, we deleted Pten in cardiac progenitor cells. We found that enhanced Akt signaling promoted SHF progenitor cell proliferation through the coordination of BMP signaling and β-catenin activity.

RESULTS

Deletion of Pten in the SHF progenitors results in SHF expansion and enlarged size of SHF derivatives

Mef2c-AHF-Cre (hereafter referred to as Mef2c-Cre) mice express the Cre recombinase in the anterior heart field (AHF), a subset of the SHF which gives rise to OFT/RV (Verzi et al., 2005). This Cre line has been extensively utilized to delete genes for investigation of the SHF development in mice (Ai et al., 2007; Bai et al., 2013; Engleka et al., 2012; Xie et al., 2012). Using Mef2c-Cre, Pten was deleted in the SHF. Staining for β-galactosidase clearly labeled the SHF and its derivatives, i.e. the OFT and the RV (Fig. 1A,B). Deletion of Pten resulted in a substantial expansion of SHF in the PM (Fig. 1A,B). The OFT and the RV in Pten deletion mice also showed significantly increased sizes compared with those of the control tissue (Fig. 1A,B). Quantitative analysis revealed that the SHF area (in the PM) and the length of the OFT in Pten-deletion mice were significantly greater than in control mice (supplementary material Fig. S1A,B). Anatomical and histological analysis of embryos at later stages displayed significantly enlarged RV and arteries in Pten
deletion mice (Fig. 1C-H). The heart function of these mice showed signs of impairment in the forms of edema (Fig. 1I). The majority of the mice died within 10 days after birth (Fig. 1S). Dead newborns were not presented with milk in the stomach or with hypertrophic heart (Fig. 1J-L). In addition, WGA staining showed no significant difference in cardiomyocyte size between 

\[ \text{Pten}^{+/-}; \text{Me2c-Cre} \]

and control mice by mid-gestational stage (E14.5) (Fig. 1M,N,Q), although at this stage cardiomyocyte proliferation was profoundly higher in 

\[ \text{Pten}^{-/-}; \text{Me2c-Cre} \]

mice than that of control mice (supplementary material Fig. S1C-K). Furthermore, we observed enlarged sizes of cardiomyocytes in 

\[ \text{Pten}^{-/-}; \text{Me2c-Cre} \]

deletion newborns compared with those of control mice (Fig. 1O-Q). Accordingly, expression of hypertrophic markers, such as \[ \text{Anp} \]

and \[ \text{Bnp} \] (\[ Nppa \] and \[ Nppb \], respectively – Mouse Genome Informatics), was not changed in the heart of 

\[ \text{Pten}^{-/-}; \text{Me2c-Cre} \]

mice at E14.5, but their levels were significantly increased in 

\[ \text{Pten}^{-/-}; \text{Me2c-Cre} \]

deletion mice at birth (Fig. 1R).

Taken together, these results indicate that PTEN inactivation affects SHF progenitors and SHF development.
\textbf{Pten} deletion promotes the proliferation of SHF progenitor cells

The enlarged SHF resulting from \textit{Pten} loss suggests enhanced cell proliferation of cardiac progenitors. Therefore, we assessed cell proliferation in the SHF. Isl1 (Isl1)-positive cells in the PM and proximal OFT indicate cardiac progenitor identity at E9.5. Thus, the proliferation of Isl1-positive cardiac progenitors in these regions was analyzed via immunofluorescence (IF) staining (Cai et al., 2003). The results indicated that there were significantly more cells expressing Isl1 and the mitotic marker Ki67 (Mki67 – Mouse Genome Informatics) in \textit{Pten} deletion embryos than in control embryos (Fig. 2A-I, N). Co-staining of phosphorylated histone H3 (pHH3) and Isl1 further confirmed these results (Fig. 2J-M, O). In addition, loss of \textit{Pten} led to more β-gal- and Isl1-double-positive cells than in control animals (supplementary material Fig. S1L).

\begin{figure}
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\caption{\textbf{Pten} deletion promotes the proliferation of SHF progenitors. (A) Schematic to display the horizontal section of E9.5 embryo. Boxed area marks the SHF for investigation (in red). (B-I) IF staining against Ki67/Isl1 of sections from E9.5 mouse embryos. Ki67/Isl1 double-positive nuclei are shown in yellow. B′-I′ are higher magnification views of the boxed areas in B-I. (J-M) IF staining against PHH3/Isl1 of sections from E9.5 mouse embryos. PHH3/Isl1 double-positive nuclei are shown in yellow (indicated by arrows). K′ and M′ are higher magnification views of the boxed areas in K and M. (N) Quantification of Ki67/Isl1 double-positive nuclei in B′-I′. (O) Quantification of PHH3/Isl1 double-positive nuclei in J-M, K′ and M′. (P) Gene expression levels examined by RT-qPCR. (Q-T) IF staining. Q′-T′ are higher magnification views of the boxed areas in Q-T.}
\end{figure}
Next, we performed real-time quantitative PCR (RT-qPCR) to examine gene expression levels in the SHF region (Fig. 2A) of control and Pten deletion embryos. As expected, the expression levels of Pten were significantly decreased in Pten deletion mice compared with control mice at E9.5 (Fig. 2P). However, we observed profoundly enhanced Isl1 expression in Pten deletion mice compared with control mice, which was consistent with increased cell proliferation of SHF progenitors (Fig. 2P). The expression levels of the key cardiac transcription factors, including Nkx2.5, Hand2, Mef2c, Gata4, Tbx5 and Tbx1, were comparable between control and Pten deletion mice (Fig. 2P). FGF8 and FGF10 are crucial signaling molecules promoting SHF progenitor proliferation, and their expression levels were not altered in Pten deletion mice (Fig. 2P). In addition, we investigated early cardiac muscle-specific genes, such as tropomyosin (Tpm) and α-smooth muscle actin (αSMA; Acta2 – Mouse Genome Informatics) by IF staining and observed similar patterns in control and Pten deletion mice (Fig. 2Q-T'). These results suggest that cardiac differentiation was nearly normal in Pten deletion mice.

PTEN is a negative genetic regulator of Akt1 signaling
PTEN is a lipid phosphatase antagonizing the PI3K signaling, which mediates several signal transduction pathways, including the mitogen-activated protein kinase (MAPK) signaling pathway and the PDK1 signaling pathway (Cantley, 2002). PDK1 phosphorylates and subsequently activates a panel of kinases, such as Akt,
PKC (Prkcg – Mouse Genome Informatics), SGK (Sgk1 – Mouse Genome Informatics) and S6K (Rps6k – Mouse Genome Informatics) (Pearce et al., 2010). Although Akt is a key downstream target that is negatively regulated by PTEN, genetic evidence is lacking regarding the relationship between PTEN and Akt during mouse embryogenesis and organogenesis.

To examine whether Akt activity is promoted in the heart tissue of Pten deletion mice, we performed IF staining in E9.5 embryonic hearts. Phosphorylation of Akt at serine 473 (S473) should indicate Akt activation, and IF staining of Akt phospho-S473 revealed a stronger signal in Pten deletion SHF than in that of controls (Fig. 3A-G).

Next, we tested the genetic relationship between PTEN and Akt during heart development. The deletion of Akt1 from Pten deletion mice generated the Pten/Akt1 double-deletion mice (Ptenf/f; Akt1f/f; Mef2c-Cre). Anatomic and histological analysis revealed comparable heart sizes between Pten/Akt1 double-deletion mice and control mice, indicating that the increased heart size of Pten deletion mice was rescued by abrogation of Akt1 (Fig. 3H-O).

Similarly, we also observed that Mesp1-Cre-mediated Pten deletion (Ptenf/f; Mesp1-Cre) caused heart defects and embryonic lethality beginning at E9.5, and complete lethality by E12.5. However, the deletion of Akt1 from Pten deletion mice generated the Pten/Akt1 double-deletion mice (Ptenf/f; Akt1f/f; Mesp1-Cre), which were able to develop to full term, with some progeny surviving for 3-4 months (supplementary material Fig. S2A-D-G). Furthermore, we found that deletion of Akt2 or Akt3 also delayed the embryonic lethality of Pten deletion mice (supplementary material Fig. S2B,C). Histological analysis revealed nearly normal heart development in Pten/Akt1 double-deletion mice, although a slightly enlarged heart was observed (supplementary material Fig. S2A).

Taken together, these results indicate that Akt1 is the key downstream target of PTEN and is negatively regulated by PTEN during heart development. The increased size of the SHF and the defective SHF development found in Pten deletion mice are a consequence of enhanced Akt activity.

PTEN-Akt signaling suppresses BMP-SMAD signaling through FOXO

BMP signaling is required for cardiac progenitor specification, proliferation and differentiation (Brand, 2003; Klaus et al., 2012; Mercola et al., 2011; Olson and Schneider, 2003; van Wijk et al., 2007). To understand how enhanced Akt activity gives rise to SHF expansion, we studied BMP signaling in the SHF of Pten deletion mice. IF staining of phospho-SMAD1/5, an indicator of the activation of BMP signaling, showed a markedly reduced signal in the SHF of Pten deletion hearts (Fig. 4A-I).

Fig. 4. PTEN-Akt signaling suppresses BMP-SMAD signaling. (A-H) IF staining against phospho-SMAD1/5 in the SHF at E9.5. (A’-H’) Higher magnification views of the boxed areas in A-H. (I) Quantification of p-SMAD1/5 nuclei in the SHF of A-H’. (J) Western blot analysis. (K,L) RT-qPCR analysis of Bmp ligands and receptors.
Western blot analysis also revealed a consistent change in phospho-SMAD1/5 (Fig. 4J; supplementary material Fig. S3A,B). Among the ∼20 Bmp ligands, Bmp2, 4, 7 and 10 are expressed in the SHF (Liu et al., 2004; Wang et al., 2010; Yuasa and Fukuda, 2008). We examined the expression levels of these Bmp ligands and their receptors in the SHF via RT-qPCR, and the results revealed significantly reduced levels of Bmp7 in Pten deletion mice compared with those of the controls (Fig. 4K). However, the expression levels of BMP receptors, including Alk1-3, Alk6 and Bmpr2, were comparable between control and Pten deletion mice (Fig. 4L).

Collectively, these data indicate that BMP signaling is dampened in the SHF of Pten deletion embryos.

Akt1 signaling was hyperactivated upon Pten deletion, and we observed nuclear localization of active Akt. Therefore, we hypothesized that the expression of Bmp 7 was suppressed by Akt signaling at the transcriptional level. The forkhead box O (FOXO) family of transcription factors are well-established Akt substrates, and Akt signaling might repress the expression of BMP signaling components through inhibition of FOXO (Evans-Anderson et al., 2008). To test this hypothesis, we first investigated the phosphorylation status of FOXO in heart tissue via IF staining. The levels of FOXO phosphorylation in the Pten deletion hearts were profoundly enhanced compared with control hearts (Fig. 5A-G). Phosphorylation of FOXO by Akt translocates FOXO from the nucleus to the cytosol. Accordingly, we observed more cytosolic phospho-FOXO in the Pten deletion hearts than in control hearts (Fig. 5A-G; and supplementary material Fig. S4). Furthermore, western blot analysis revealed significantly enhanced FOXO phosphorylation levels in the heart of Pten deletion mice compared with control mice (Fig. 5H).

To test whether FOXO activates the transcription of Bmp7, we ligated a luciferase reporter into the upstream cis-elements of the Bmp7 gene, which contains a FOXO-binding consensus sequence (Fig. 5I). This luciferase reporter assay showed that constitutively active FOXO1 (FOXO1-AAA) strongly activated the transcription...
of the luciferase reporter, whereas mutation of the FOXO-binding consensus sequence blocked this activation (Fig. 5I,J).

Taken together, these results demonstrate that Akt signaling suppresses BMP signaling through phosphorylation and inhibition of FOXO.

**PTEN-Akt signaling regulates β-catenin activity**

Wnt signaling regulates early cardiogenesis and is involved in SHF development. Deletion of the Wnt effector β-catenin in the SHF caused impaired RV formation (Kwon et al., 2007). It was previously reported that phosphorylation of β-catenin at the C-terminal S552 by Akt promotes β-catenin nuclear localization and activation in ISCs (He et al., 2007). We first investigated β-catenin phosphorylation levels in heart tissues through western blot analysis and found much higher phospho-β-catenin levels in the hearts of Pten−/−; Mef2c-Cre mice than in control hearts (Fig. 6A). Pten−/−; Akt1+/+; Mesp1-Cre mice also showed strong phosphorylation of β-catenin in the heart (Fig. 6B).

Next, we tested whether reducing β-catenin activity could reverse the phenotype of Pten deletion mice. We found that partial deletion of β-catenin partially reduced the heart size of Pten deletion mice (Fig. 6C-L) and also decreased cell proliferation of the SHF progenitors of Pten deletion mice (Fig. 6M-P).

Collectively, these results showed that β-catenin activity was elevated in Pten deletion mice, which disrupted heart development.
Reducing Akt signaling in SHF progenitors

PDK1 is indispensable for Akt activation during heart development (Feng et al., 2010). We deleted Pdk1 in the SHF and observed impaired development of the RV and the OFT (Fig. 7A-J). IF staining of phospho-SMAD1/5 revealed an increased signal in the hearts of Pdk1 deletion mice compared with the controls (Fig. 7K-S).

DISCUSSION

Our findings provide new insights into SHF development, which is a fundamental, but poorly understood, developmental event that governs OFT formation and ventricular expansion in mammals. We found that enhancing Akt signaling through Pten deletion caused expansion of the SHF and overgrowth of SHF derivatives, the OFT and the RV, whereas the inhibition of Akt signaling upon PDK1 and Akt inactivation results in a smaller OFT and RV. Thus, our findings demonstrate a crucial role of Akt signaling in SHF development.

Furthermore, we uncovered a mechanism underlying the coordination of BMP and Wnt-β-catenin signaling to regulate SHF proliferation and differentiation (supplementary material Fig. S5A).

Genetic relationship between PTEN and Akt1 in SHF development

One property of SHF progenitors is continued cell proliferation (Watanabe et al., 2012). FGF signaling drives the cell proliferation of SHF progenitors. Among the numerous FGF ligands, FGF8 and 10 are important regulators involved in SHF development. FGF10 was the first identified molecular marker of the murine SHF, and mouse genetic studies have revealed that both FGF8 and FGF10 promote SHF progenitor proliferation through activation of the MAPK/ERK pathway (Watanabe et al., 2010). In a variety of mouse and human tissues, FGFs exert their functions by modulating two pivotal signaling pathways: the MAPK/ERK signaling pathway and the PI3K-Akt signaling pathway. Therefore, FGF ligands, including FGF4, 8 and 10, are most likely the upstream intercellular growth factors that activate PI3K-Akt signaling in the SHF.

There are three Akt proteins in mice and humans, Akt1, 2 and 3, and previous studies by our group have demonstrated that all of these proteins are expressed in embryonic heart tissues (Chang et al., 2010). Although deletion of Akt1 is sufficient to suppress tumor development in Pten−/− mice, genetic evidence of the relationship between PTEN and Akt1 during mouse embryonic development is lacking. In this study, we demonstrated that Akt1 is the key downstream target that is negatively regulated by PTEN, and we established the first genetic relationship between PTEN and Akt1 in the SHF and heart development.

Pten is expressed throughout embryogenesis, and germline deletion of Pten causes embryonic lethality by E9.5, indicating that PTEN is essential for mouse embryonic development (Suzuki et al., 1998). Thus, it is possible that modulation of Pten expression or PTEN protein levels by signals other than FGF signaling impacts the SHF development.
Crosstalk between PTEN-Akt signaling and BMP signaling

Previously, Li and colleagues reported that BMP suppresses PTEN phosphorylation that inactivates PTEN, resulting in enhanced PTEN activity in ISCs (He et al., 2004). Their results indicate that BMP suppresses Akt activity through PTEN in ISCs. In this study, we found that Akt signaling represses the expression of Bmp ligand (Bmp7) in SHF progenitors. As a result, BMP signaling is reduced by Akt activation. There might be a reciprocal antagonizing effect between Akt and BMP signaling in SHF development. Whether BMP inhibits Akt signaling in the SHF needs further investigation.

Regulation of the stem cell/progenitor cell switch from quiescent to active state

Based on their own and others’ research on stem cells, Clevers and Li proposed a model for the switch of stem cells from a quiescent state to an active state (Li and Clevers, 2010). In the quiescent state, BMP signaling is active, and Wnt signaling is turned off in stem cells. Once stem cells are activated for proliferation (the active state), BMP signaling is shut off and Wnt signaling is switched on. Nevertheless, how the switch from a quiescent/BMP-on state to an active/Wnt-on state is regulated remains elusive. Our findings suggest that Akt signaling is a mediator coordinating both BMP and Wnt signaling during the transition state of stem/progenitor cells (supplementary material Fig. S5B). In the future, it will be intriguing to test this hypothesis in adult stem cells, such as hair follicle, gut and bone marrow stem cells.

We observed enlarged LVs of Pten deletion mice from late gestational stage. Lineage tracing showed that Pten-deletion cardiomyocytes moved from the RV to the LV, suggesting that the mutant cardiomyocytes have gained migratory ability or dominated the RV due to increased proliferation. This hypothesis deserves further investigation.

MATERIALS AND METHODS

Mice

The previously described mouse strains used in this study included Pten conditional mice (Suzuki et al., 2001), Mesp1-Cre and Mef2c-AHF-Cre mice (Klaus and Birchmeier, 2009; Saga et al., 1999; Verzi et al., 2005), β-catenin-floxed mice (Huelsken et al., 2001) and Akt1 and Pdk1 conditional mice (Di et al., 2012; Feng et al., 2010; Zhao et al., 2014). All mouse lines were maintained in a B6 genetic background. The experimental animal facility has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and the Institutional Animal Care and Use Committee (IACUC) of Model Animal Research Center of Nanjing University, China, approved all animal protocols used in this study.

Histological analysis and β-galactosidase staining

Mouse embryos were collected and fixed in 4% paraformaldehyde, then dehydrated, embedded in paraffin and sectioned. Histological sections were stained with hematoxylin and eosin (H&E) or used for other analyses, such as immunohistochemistry (IHC), immunofluorescence (IF) staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. For β-galactosidase staining, embryos were harvested and pre-fixed for 2 h in 4% paraformaldehyde on ice, and β-galactosidase staining was subsequently performed as previously described (Prall et al., 2007).

IF and IHC staining

Embryos were fixed in 4% paraformaldehyde for no more than 2 h. After fixation, the embryos were rinsed in PBS and incubated in 30% sucrose at 4°C for at least 8 h. The embryos were then embedded in OCT medium in the required direction. The embedded embryos were snap-frozen in liquid nitrogen and stored at −80°C. Sections were cut at a thickness of 7-10 μm. Subsequently, the sections were blocked with 10% goat serum and incubated with appropriately diluted primary antibodies at 4°C overnight. Afterwards, the sections were washed three times in PBS for 5 min each and then incubated with secondary antibodies or other dyes at room temperature for 2 h. The fixed and stained sections were mounted with nail polishing oil for analysis. IHC staining was carried out according to manufacturer’s recommendations and detected with 3,3-diaminobenzidine (DAB) (Maixin-Bio). The following antibodies were used for IF staining: anti-p-Akt (Cell Signaling Technology, CST 4691; 1:200), anti-p-Smad1/5 (Cell Signaling Technology, CST 9151; 1:200), anti-β-catenin S552 (Cell Signaling Technology, CST 5651S; 1:200), anti-Ki67 (DakoCytomation, M724901; 1:200), anti-p-Histone H3 (Cell Signaling Technology, CST 9701S; 1:200), anti-α-SMA (Sigma, c6198; 1:200), anti-p-FOXO (Cell Signaling Technology, CST 2880; 1:200), and anti-Tropomyosin (1:200) from the Developmental Studies Hybridoma Bank (DSHB; University of Iowa, Iowa City, USA).

Western blot analysis

Embryonic hearts were harvested, snap-frozen in liquid nitrogen and stored until use. Tissue lysates were prepared in lysis buffer as previously described (Chang et al., 2010). Approximately 50 μg of protein per sample was separated via electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked with TBST (50 mM Tris, 150 mM NaCl, 0.5 mM Tween 20, pH 7.5) and incubated with primary antibodies overnight. The samples were analyzed via immunoblotting with antibodies against t-Akt (BioWorld, BS1810; 1:1000), p-Akt (Cell Signaling Technology, CST 4060; 1:1000), Smad1 (Cell Signaling Technology, CST 6944; 1:500), p-Smad1/5 (Cell Signaling Technology, CST 9516; 1:500), β-catenin252 (Cell Signaling Technology, CST 5651S; 1:500) and anti-GAPDH (BioWorld, AP0063; 1:20,000). The membranes were then incubated with a secondary anti-rabbit or anti-mouse HRP-conjugated antibody, and the resulting signals were detected through enhanced chemiluminescence (ECL).

RT-qPCR

Total RNA from embryonic hearts or pharyngeal arches was isolated with TRIzol reagent (Invitrogen), and 1 μg of RNA was reverse-transcribed using a reverse transcription system kit (Promega). RT-qPCR was performed using an ABI7900 thermocycler with the Roche SYBR Green RT-qPCR reagent. The expression of selected genes involved in cardiac development was examined, and the results were normalized to the expression of GAPDH.

Plasmid construction and luciferase reporter assay

A ∼3.4 kb fragment, corresponding to the Bmp7 5’ flanking sequence, was amplified via PCR from mouse genomic DNA and cloned into the pGL3-Basic Vector (Promega) to generate the Bmp7-luc vector. The Bmp7 mutant luc vector, which contains a 2-bp mutation in the mouse Bmp7 FHRE box (AA to CC), was generated using the Mutabest kit (Takara). Luciferase assays were performed using the Dual Luciferase Assay System (Promega), following a standard protocol. At least ten independent transfections were conducted, and all assays were performed three times.

Statistical analysis

All results are presented as mean±s.e.m. Statistical analyses were performed using Student’s t-test with GraphPad Prism 4.03 software. Multiple groups were tested via one-way ANOVA, and comparisons between two groups were performed using Student’s t-test. A P-value of <0.05 was considered significant (*), and P-values of <0.01 (**) or <0.005 (***) were considered highly significant.

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Competing interests

The authors declare no competing or financial interests.
Author contributions W.L. and Z.Y. conceived and designed the experiments; W.L., X.Z., L.T., J.Z., H.W. and H.J. performed experiments and analyzed the data; W.L. and Z.Y. wrote the manuscript; W.L., X.Z., L.T., J.Z., H.W., H.J. and Z.Y. discussed and reviewed the manuscript.

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