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Myocardial *Smad4* Is Essential for Cardiogenesis in Mouse Embryos

Lanying Song, Wensheng Yan, Xinbin Chen, Chu-xia Deng, Qin Wang, and Kai Jiao

Department of Genetics (L.S., K.J.), Division of Genetic and Translational Medicine; Departments of Cell Biology (W.Y., X.C.) and Physiology (Q.W.), The University of Alabama at Birmingham. Genetics of Development and Disease Branch (C.-x.D.), National Institute of Diabetes and Digestive and Kidney, NIH, Bethesda, Md.

Abstract

Congenital heart diseases are the most commonly observed human birth defects and are the leading cause of infant morbidity and mortality. Accumulating evidence indicates that transforming growth factor- β /bone morphogenetic protein signaling pathways play critical roles during cardiogenesis. *Smad4* encodes the only common Smad protein in mammals, which is a critical nuclear mediator of transforming growth factor- β /bone morphogenetic protein signaling. The aim of this work was to investigate the roles of *Smad4* during heart development. To overcome the early embryonic lethality of *Smad4*^{-/-} mice, we specifically disrupted *Smad4* in the myocardium using a Cre/loxP system. We show that myocardial-specific inactivation of *Smad4* caused heart failure and embryonic lethality at midgestation. Histological analysis revealed that mutant mice displayed a hypocellular myocardial wall defect, which is likely the primary cause for heart failure. Both decreased cell proliferation and increased apoptosis contributed to the myocardial wall defect in mutant mice. Data presented in this article contradict a previous report showing that *Smad4* is dispensable for heart development. Our further molecular characterization showed that expression of *Nmyc* and its downstream targets, including cyclin D1, cyclin D2, and *Id2*, were downregulated in mutant embryos. Reporter analysis indicated that the transcriptional activity of the 351-bp *Nmyc* promoter can be positively regulated by bone morphogenetic protein stimulation and negatively regulated by transforming growth factor- β stimulation. Chromatin immunoprecipitation analysis revealed that the *Nmyc* promoter can form a complex with Smad4, suggesting that *Nmyc* is a direct downstream target of Smad4. In conclusion, this study provides the first mouse model showing that *Smad4* plays essential roles during cardiogenesis.

Keywords

Smad4; cardiogenesis; transforming growth factor β /bone morphogenetic protein; *Nmyc*; myocardium

Congenital heart diseases (CHDs) are the most commonly observed human birth defects and are the leading cause of infant morbidity and mortality,^{1,2} yet their molecular etiologies are poorly understood. Accumulating evidence from mouse genetic studies has suggested that members of the family of transforming growth factor (TGF) β /bone morphogenetic protein (BMP) cytokines play critical functions during heart development.³⁻⁵ Mouse embryos derived from epiblasts lacking *Bmp1ra* do not contain any observable heart rudiment, suggesting that BMP signaling is required for induction of heart formation from mesoderm.^{6,7} Myocardial depletion of *Alk3* or inactivation of *BMP10* causes heart failure and embryonic lethality at midgestation because of significantly elevated apoptosis and reduced proliferation in cardiomyocytes.^{8,9} Myocardial inactivation of *BMP2* leads to abnormal specification of the atrioventricular canal (AVC) myocardium and failure of atrioventricular (AV) cushion formation.^{10,11} *TGF β 2*^{-/-} embryos display a range of cardiac defects including double-outlet right ventricle, atrial or ventricular septal defect, and an overriding tricuspid valve.^{12,13} Myocardial expression of a constitutively active form of *Tgfb1* disrupts cardiac looping and leads to heart failure.¹⁴ Therefore TGF β /BMP signaling plays critical roles during multiple steps and in many aspects of cardiogenesis.

TGF β /BMP signals are transduced through heterodimeric complexes of type I and type II serine/threonine kinase receptors. After formation of the receptor/ligand complex, the type II receptor will phosphorylate the type I receptor, which in turn phosphorylates specific members of the receptor-activated Smads (R-Smads).¹⁵⁻¹⁸ Phosphorylated R-Smads associate with the co-Smad Smad4, and the complex translocates to the nucleus to regulate transcription of target genes. In addition to the “canonical” pathway of Smad-mediated transcription, TGF β /BMP signaling may also transduce signals through “noncanonical” mitogen-activated protein kinase pathways.^{17,19}

Smad4 encodes the only co-Smad in mammals that can interact with both BMP and TGF β R-Smads. Although Smad4 was originally thought to be an essential component of all Smad transcriptional complexes, recent studies have shown that TGF β /BMP-activated transcription of some genes can occur in the absence of Smad4.^{20,21} Furthermore, *Smad2*^{-/-} embryos display more severe defects than *Smad4*^{-/-} embryos.^{22,23} These data suggest that rather than acting as a general transcriptional coactivator of R-Smads, Smad4 mediates specific cellular responses induced by TGF β /BMP signaling.

The aim of this study was to reveal specific functions of *Smad4* during cardiogenesis. Inactivation of *Smad4* using a conventional gene knockout approach leads to defective gastrulation, precluding investigation of its roles during heart development.^{22,24} Specific inactivation of *Smad4* in epiblasts causes embryonic lethality at embryonic day (E)8.5; however, in mutant embryos, heart rudiments are formed, indicating that *Smad4* is not required for induction of cardiomyocytes from lateral mesoderm.²³ Recent work by Wang et al reported no observable embryonic heart defect following myocardial-specific inactivation of *Smad4*, suggesting that *Smad4* is dispensable for heart development.²⁵ In contrast to this published work, our study provides compelling mouse genetic evidence showing that *Smad4* plays essential cardiogenic roles, because myocardial inactivation of *Smad4* leads to heart failure and embryonic lethality at midgestation. We have further performed a detailed characterization of the novel mouse model at both the morphological and molecular levels.

Materials and Methods

Mouse and Embryo Manipulations

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Alabama. The *cTnt-Cre;Smad4^{loxP/+}* mice were obtained by crossing the *cTnt-Cre* mice²⁶ with the *Smad4^{loxP/loxP}* mice,²⁷ both of which were backcrossed with C57Bl6 mice for more than 5 generations. Embryo treatment was performed as described previously.^{28,29} The PCR conditions for amplifying *Cre* and unrecombined and recombined *Smad4^{loxP}* alleles have been described previously.^{26,27}

TUNEL, Nonradioactive Section In Situ Hybridization, and Western Blot Analysis

TUNEL assays were performed using the Dead End Colorimetric TUNEL system (Promega) following the instructions of the manufacturer. Nonradioactive section in situ hybridization and Western blot analysis were performed as described previously.^{29,30}

Immunostaining Studies

Immunofluorescence studies were performed as described previously.^{28,29} Samples were examined with the Leica HC fluorescent microscope equipped with an RT SLIDER digital camera. Immunohistochemistry studies were performed using the Envision+ system (DakoCytomation). The primary antibodies used in this study included antibodies recognizing cyclin D1 (BD Biosciences), cyclin D2 (Santa Cruz Biotechnology), phospho-H3 (Upstate), Smad4, phospho-Smad1/5/8, phospho-Smad2/3 (Cell Signaling), NFATc1 (BD Biosciences), cardiac myosin heavy chain, cardiac troponin T (Iowa Hybridoma Bank), and p57^{Kip2} (Labvision).

Luciferase Reporter Analysis

The 351-bp promoter region of the mouse *Nmyc* gene was PCR amplified and cloned into the pGL2-basic vector to acquire the pNmyc-Luc construct. Site-directed mutagenesis was performed to disrupt the potential Smad4-binding site within the *Nmyc* promoter using the QuickChange II site-directed mutagenesis kit (Stratagene). The mutagenic primer sequence is 5'-CAAAGCGCAGCCAGTGACAGTCATCTCCGGATCCCGCGCTGGGTGGATGCG, in which the 7 underlined bases are the mutations that disrupt the potential Smad4-binding site. Transient transfection was performed with Fugene6 (Roche) following the instructions of the manufacturer. Treatment of cells with hBMP4 and TGF β ₁ (R&D) and measurement of luciferase activity were performed as previously described.^{30,31}

Chromatin Immunoprecipitation Analysis

P19 cells were transfected with the pCMV-HA vector or pHA-Smad4, which expresses hemagglutinin (HA)-tagged Smad4.³⁰ Chromatin immunoprecipitation (ChIP) analysis was performed as described previously³¹ using an anti-HA antibody (Covance). Rabbit IgG was used as a negative control. The 351-bp *Nmyc* promoter region was amplified with the forward primer 5'GAGAAAAG-CAAATGGCTTTTGGC and the reverse primer 5'-TCTCCGGGTGGGCTGAGGGAG An unrelated genomic DNA sequence (corresponding

to the DNA sequence at the *Tgfb β 2* allele) was amplified with primers 5'TAAACAAGGTCCGGAGCCCA and 5'ACTTCTGCAAGAGGTCCCCT

Results

Inactivation of *Smad4* in the Myocardium by *cTnt-Cre*

To reveal cardiogenic roles of *Smad4* and to overcome the early lethality of *Smad4*^{-/-} mouse embryos,²²⁻²⁴ we specifically inactivated *Smad4* in the myocardium by crossing the male *cTnTcre;Smad4*^{lox β /+} mice with the female *Smad4*^{lox β /lox β} mice.²⁷ The *cTnt-Cre* induces recombination early in the cardiomyocyte lineage (E7.5) and efficiently inactivates target genes between E9.5 to E10.5.^{26,32,33} To confirm that *Smad4* was efficiently inactivated in the myocardium, we performed PCR analysis using genomic DNA isolated from E10.5 embryos (Figure 1A and 1B). The signal for the unrecombined *Smad4*^{lox β} allele in mutant hearts was reduced to less than 10% of the control level. As expected, the recombined allele was detected in only mutant hearts and not in control samples (*Smad4*^{lox β /lox β}). To evaluate the reduction of Smad4 at the protein level, we performed immunohistochemistry analysis using a Smad4-specific antibody. Expression of Smad4 in the myocardium was dramatically reduced in mutant hearts, yet Smad4-positive cells were evident in the mutant AV cushions (Figure 1C through D'), confirming that *cTnt-Cre* specifically inactivated *Smad4* in cardiomyocytes.

To test whether depletion of *Smad4* in the myocardium affects activation of BMP and TGF β R-Smads, we performed immunohistochemistry analysis using antibodies against phospho-Smad1/5/8 or against phospho-Smad2/3 (Figure I in the online data supplement, available at <http://circres.ahajournals.org>). No obvious alteration in the level of activated R-Smads was observed in mutant hearts, consistent with the previously reported observation that activation of R-Smads is independent of the presence of Smad4.^{17,18}

Requirement of *Smad4* During Normal Cardiogenesis in Mouse Embryos

We did not recover any mutant (*cTnt-Cre;Smad4*^{lox β /lox β}) neonates from the breeding of *cTnTcre;Smad4*^{lox β /+} mice and *Smad4*^{lox β /lox β} mice (total of 7 litters), suggesting that myocardial inactivation of *Smad4* causes embryonic lethality. To determine the stage at which embryonic death occurred, we isolated embryos from E9.5 to E16.5. The number of mutant embryos between E9.5 and E11.5 was not markedly different from the expected ratio (25%, supplemental Figure II, A). At the E12.5 stage, the number of living mutant embryos was reduced to \approx 10% of total embryos, and very few mutant embryos survived beyond E13.5, indicating that the majority of embryonic lethality occurred between E11.5 and E13.5. At E12.5, all mutant embryos (including those that were alive) displayed internal hemorrhages (supplemental Figure II, B), suggesting that the embryonic lethality was caused by cardiovascular insufficiency.

To reveal heart defects caused by myocardial depletion of *Smad4*, we performed detailed histological examination of living embryos between E9.5 and E12.5. No obvious abnormality was observed in E9.5 and E10.5 mutant hearts (Figure 2). At E11.5, although the AV cushions were of normal size, the superior and inferior AV cushions were

improperly aligned in mutant hearts. The myocardium of mutant embryos at E11.5 does not display any obvious morphological defects except that the number of cells in the trabeculation zone appeared to be higher in mutant hearts than in those of controls (Figure 2C and 2G). At E12.5, all mutant hearts displayed thin myocardial walls (Figure 2D, 2D', H, and H'), suggesting that *Smad4* is required for the proper morphogenesis of myocardial wall. The hypocellular myocardial wall can reduce contractility of embryonic hearts, resulting in cardioinsufficiency, and is likely the primary cause of the embryonic lethality observed in mutants. Despite the obvious misalignment defect between the superior and inferior AV cushions in E11.5 hearts, AV cushions in E12.5 mutants were properly fused to form the central mesenchymal mass, which correctly separated the AV canal into left and right channels. The intraventricular septum in mutant hearts was formed at the proper position separating left and right ventricles (E12.5), although it was thinner than that in controls (supplemental Figure II).

Molecular Examination of Mutant Embryonic Hearts

To determine the molecular defects in mutant (*cTnt-Cre;Smad4^{loxP/loxP}*) embryonic hearts, we studied expression of multiple genes that play critical roles during cardiogenesis. We first examined general cardiogenic transcription factors including *Nkx2.5*, *Mef2c*, *GATA4*, and *myocardin* and showed that they were properly expressed in mutant embryos (Figure 3A and 3B; supplemental Figure III, A through F). Expression of *Chisel*, which was downregulated by *BMP10* inactivation, was not altered in mutant hearts (supplemental Figure III, G and H). Furthermore, myocardial cells in mutants appear to be appropriately differentiated, as judged from normal expression of cardiomyocyte differentiation markers including cardiac troponin T (Figure 3C and 3D) and cardiac myosin heavy chain (supplemental Figure III, I and J). These data are consistent with previous results indicating that inactivation of *Smad4* in epiblasts did not block specification and differentiation of cardiomyocytes.²³ To test whether the AVC myocardium was properly specified, we examined expression of *BMP2* and *Tbx2* and found no abnormal expression of the 2 genes in mutant hearts (Figure 3E and 3F; supplemental Figure III, K and L), suggesting that the AV cushion misalignment defect was not caused by malspecification of the AVC region. We next examined expression of *BMP10* and *ANF* and found that expression of both trabecular markers^{9,34} was increased in mutant hearts (Figure 3G and 3H; supplemental Figure III, M and N). These findings are consistent with our observation that mutant embryonic hearts contain more cells in their trabecular zones than do hearts from control embryos. We further showed that expression of *NFATc1*^{35–38} was not altered in mutant hearts (supplemental Figure III, O and P), indicating that specification of endocardial cells was not compromised by myocardial depletion of *Smad4*.

Decreased Cell Proliferation and Increased Apoptosis in Mutant Embryonic Hearts

To reveal the cellular mechanism accounting for the hypocellular myocardial wall defect observed in mutant hearts, we first examined cell proliferation by performing immunofluorescence studies using a primary antibody against phospho-H3. No reduction in cell proliferation was observed in mutant hearts until E10.5 (data not shown). In E11.5 mutant hearts, the cell proliferation rate was reduced in ventricles but not in other heart

segments (Figure 4A through 4D), and quantitative analysis confirmed the statistical significance of the reduction ($P < 0.01$, Figure 4E).

We performed TUNEL assays to test the potential contribution of cell apoptosis to the thin-wall defect. We did not observe any obvious increase in cell death in mutant hearts until E11.5 (Figure 4F through G'). At E12.5, many cardiomyocytes in mutant hearts were undergoing apoptosis, whereas very few cardiac cells in control hearts were positive for TUNEL signals. Similar results were acquired from immunostaining studies using an anti-caspase 3 antibody (data not shown). We concluded that both decreased cell proliferation and increased apoptosis contribute to the hypocellular myocardial wall defect in mutant embryos.

Expression of *Nmyc* Is Downregulated in Mutant Hearts

We noticed that the thin-myocardial-wall defect of *cTnt-Cre;Smad4^{loxP/loxP}* embryonic hearts is similar to the cardiac defects observed in embryos with compound heterozygous *Nmyc* alleles (*Nmyc^{9a/BRP}*).³⁹ Therefore, we decided to test whether expression of *Nmyc* is altered by myocardial inactivation of *Smad4*. At E10.5, expression of *Nmyc* was weak in both wild-type and mutant embryonic hearts, and no obvious reduction in *Nmyc* expression was observed in mutant embryos (Figure 5A and 5B). At E11.5, *Nmyc* expression in mutant hearts was dramatically reduced compared with that in control hearts (Figure 5C and 5D). To further investigate these findings, we performed Western blot analysis using an anti-*Nmyc* antibody and confirmed that cardiac expression of the *Nmyc* protein was reduced in mutants (Figure 5E).

Smad4 Directly Activates Transcription of *Nmyc*

To test the possibility that Smad4 directly activates transcription of *Nmyc*, we analyzed the promoter regions of human and mouse *Nmyc* genes. Sequence alignment with the zPicture program (<http://zpicture.dcode.org>) revealed that the 351-bp promoter region (from bp-303 to bp +48, relative to the transcriptional start site) of *Nmyc* is highly conserved between human and mouse genes (supplemental Figure IV). Further sequence analysis using the rVista2.0 program (<http://rvista.dcode.org>) identified a potential Smad4-binding site within the 351-bp promoter region, which is 100% conserved between mouse and human genes. To test whether this 351-bp promoter region can respond to BMP/TGF β stimulation, we generated an *Nmyc*-luciferase reporter construct and performed luciferase analysis. Our results showed that BMP4 stimulated, whereas TGF β ₁ repressed, the transcriptional activity of the 351-bp *Nmyc* promoter in a dose-dependent manner (Figure 6A and 6B). Furthermore, the mutation disrupting the Smad4-binding site blocked the BMP4-induced upregulation of the reporter (Figure 6C), suggesting that the BMP ligand acts through the potential Smad4-binding site to simulate the *Nmyc* promoter. To test whether Smad4 directly binds to the 351-bp promoter region of *Nmyc*, we performed ChIP analysis using P19 cells transiently transfected with a plasmid expressing HA-Smad4. HA-Smad4 is specifically associated with the 351-bp *Nmyc* promoter but not with an unrelated genomic DNA sequence (Figure 6D).

Reduction of Nmyc Target Genes in Mutant Embryonic Hearts

The Nmyc gene promotes cell proliferation through activation of target genes involved in cell cycle control.^{40,41} To test whether myocardial inactivation of *Smad4* reduced expression of genes located downstream of Nmyc, we examined expression of 3 known Nmyc target genes including Id2,^{42,43} cyclin D1,⁴⁴ and cyclin D2.^{45,46} We first performed Western blot analysis using proteins extracted from wild-type and mutant embryonic hearts (E11.5) and found that expression of all 3 proteins was reduced in mutants (Figure 7A). The reduction in expression of these genes was further confirmed with immunofluorescence studies for cyclins D1 and D2 and with in situ hybridization analysis for Id2 (Figure 7). These data suggest that expression of at least a subset of Nmyc target genes was downregulated by myocardial depletion of *Smad4*. It was previously demonstrated that inactivation of *BMP10* increased expression of p57^{Kip2}, which negatively regulates the cell cycle⁹; however, we did not observe upregulation of p57^{Kip2} in *cTnt-Cre;Smad4^{loxp/loxp}* embryonic hearts (E11.5; supplemental Figure III, Q and R).

Discussion

This study provides the first mouse model demonstrating that *Smad4* plays essential roles during mammalian heart development, myocardial depletion of *Smad4* causes severe heart defects, and embryonic lethality at midgestation. Our results contradict a previously published study²⁵ applying a similar conditional gene-activation approach to address functions of *Smad4* during cardiogenesis. In that study, Wang et al inactivated *Smad4* in the myocardium using an *MHC-Cre* line and showed that mutant mice can survive to adulthood without obvious congenital defects.²⁵ The disparity between the 2 studies is unlikely caused by different mouse genetic backgrounds, as both our group and Wang and colleagues performed gene-conditional inactivation experiments on the C57BL/6 congenic background. We speculate that the discrepancy may be explained by the fact that 2 different Cre lines were used in these studies. The *cTnt-Cre* line used in this study can induce recombination of target genes starting from E7.5 and efficiently inactivate target genes at E10.5.^{26,32} We confirmed that *Smad4* was efficiently inactivated in mutant embryonic hearts at both the DNA and protein levels at E10.5. The efficiency and specificity of *Smad4* inactivation in mouse embryos using the *MHC-Cre* line generated by Wang et al were not clearly demonstrated.²⁵ Therefore, inactivation of *Smad4* with *MHC-Cre* used in the previous study may miss the critical time window in which the activity of *Smad4* is essential for heart development.

The hypocellular myocardial wall defect at E12.5 in *cTnt-Cre;Smad4^{loxp/loxp}* embryos is likely the major cause of embryonic lethality. Reduction in cell proliferation was initially observed at E11.5, when no abnormal apoptosis was detected, thus representing the primary cellular defect causing the hypocellular wall abnormality. Increased apoptosis at a later stage would further enhance this thin-myocardial-wall defect. Motivated by the similar cardiac phenotype observed with *cTnt-Cre;Smad4^{loxp/loxp}* and *Nmyc^{9a/BRP}*³⁹ embryos, we examined expression of *Nmyc* to gain further insight into the mechanism of *Smad4* regulation of myocardial cell proliferation. Our data demonstrated that expression of *Nmyc* and its target genes, including cyclin D1, cyclin D2, and Id2, were all reduced in *cTnt-Cre;Smad4^{loxp/loxp}*

embryonic hearts, suggesting that *Nmyc* plays an important role in mediating *Smad4*-promoted myocardial cell proliferation. In further support of this idea, significant reduction in cardiomyocyte proliferation was observed primarily in the ventricles, the main cardiac segment where *Nmyc* is expressed (Figure 5 and Moens et al³⁹). We observed a slightly increased number of cells in the trabeculation area of mutant hearts (Figure 2). This phenotype was repeatedly observed and was confirmed with molecular marker examination including *BMP10* and *ANF* (Figure 3; supplemental Figure III). The mechanism underlying this defect warrants further investigation.

We provide strong evidence suggesting that *Nmyc* is a direct downstream target of *Smad4* through sequence comparison, reporter analysis, and ChIP assays. This study is among the first to support that *Myc* genes can be directly upregulated by BMP stimulation. A recent study reported that *Smad1* (a BMP R-Smad) can interact with β -catenin to upregulate expression of *c-Myc*.⁴⁷ It is currently unclear whether upregulation of *Nmyc* by *Smad4* also involves β -catenin. We conclude that in addition to the indirect regulation of *Nmyc* expression by BMP signaling through *Tbx* genes,⁴⁸ BMP ligands may also directly stimulate *Nmyc* transcription through *Smad4*.

Because myocardial depletion of *Smad4* downregulates *Nmyc* expression and reduces myocardial cell proliferation, we propose that *Smad4* primarily mediates BMP signaling, rather than $TGF\beta$ signaling, during cardiogenesis. Among mouse models with mutations in BMP ligand genes, the myocardial wall defect of *cTnt-Cre;Smad4^{loxp/loxp}* embryos most closely resembles *BMP10^{-/-}* embryos, which also displayed reduced myocardial cell proliferation.⁹ However, depletion of *BMP10* also dramatically decreased expression of some general cardiogenic transcription factors including *Mef2c* and *Nkx2.5* (and its downstream target, *Chisel*) and increased expression of *p57^{kip2}*.⁹ These defects were not observed in *cTnt-Cre;Smad4^{loxp/loxp}* embryos. Therefore, the activities of *BMP10* to promote expression of cardiogenic transcription factors and to repress expression of *p57^{kip2}* are mediated by *Smad4*-independent pathways.

In conclusion, this study provides convincing mouse genetic evidence showing that *Smad4* is required for normal cardiogenesis. Functions of myocardial *Smad4* are summarized in Figure 8. Our data support the notion that $TGF\beta$ /BMP signaling in the myocardium can be mediated through both *Smad4*-dependent and -independent pathways. Furthermore, we show that *Nmyc* and its downstream genes play major roles in mediating functions of *Smad4* during cardiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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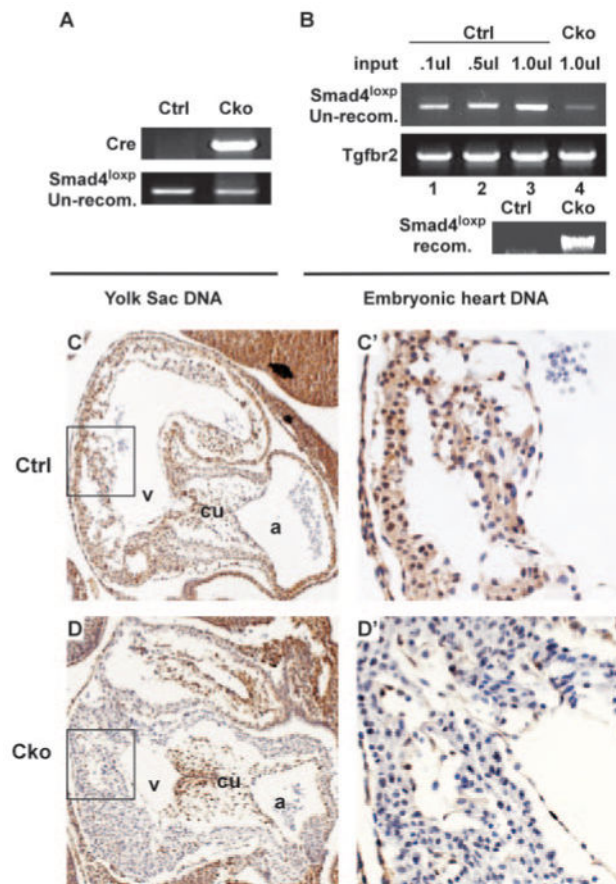


Figure 1.

Myocardial-specific inactivation of *Smad4*. A, Genomic DNA was extracted from yolk sacs of mouse embryos at E10.5 to determine their genotypes using PCR analysis with primers for Cre or for the unrecombined *Smad4^{loxp}* allele. B, Genomic DNA was extracted from hearts of the control (*Smad4^{loxp/loxp}*) and mutant (*cTnt-Cre;Smad4^{loxp/loxp}*) embryos examined in A. Semiquantitative PCR analysis was performed with primers for the unrecombined *Smad4^{loxp}* allele (top). Lanes 1 to 3 are control samples with 10%, 50%, and 100% of input DNA. Lane 4 is the mutant sample with 100% of input DNA. *Tgfb2* (an unrelated genomic DNA fragment) was used as a loading control (middle). Bottom, genomic DNA was subjected to PCR analysis using primers for the recombined *Smad4^{loxp}* allele. The PCR product could be detected in only the mutant sample and not in the control. C through D', Sections of control (C and C') and mutant (D and D') mouse hearts at E10.5 were immunostained with an anti-Smad4 antibody (brown). Samples were counterstained with hematoxylin (blue). C' and D' correspond to the boxed regions of C and D, respectively. Cko indicates *cTnt-Cre;Smad4^{loxp/loxp}*; Ctrl, *Smad4^{loxp/loxp}*; Cu, cushion.

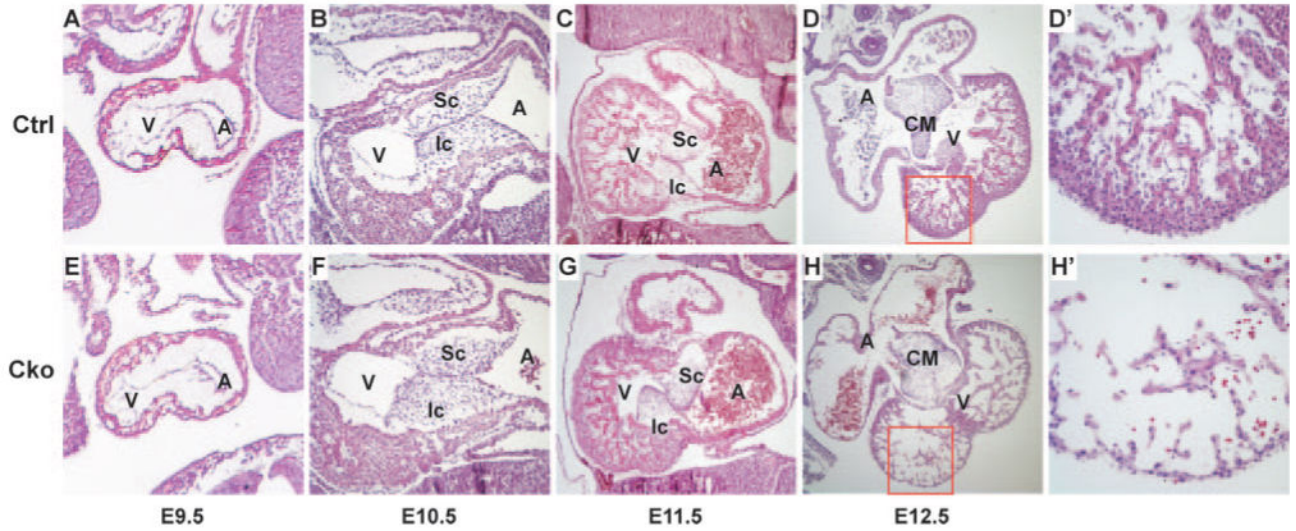


Figure 2.

Myocardial depletion of *Smad4* causes heart defects. Control (A through C) and mutant (E through G) embryos at E9.5 to E11.5 were sagittally sectioned and hematoxylin and eosin stained. Control (D and D') and mutant (H and H') embryonic hearts at E12.5 were cross sectioned and hematoxylin and eosin stained. D' and H' correspond to the boxed regions of D and H, respectively. A indicates atrium; CM, central mesenchymal mass; Cko, *cTnt-Cre;Smad4^{loxp/loxp}*; Ctrl, *Smad4^{loxp/loxp}*; Ic, inferior AV cushion; Sc, superior AV cushion; v, ventricle.

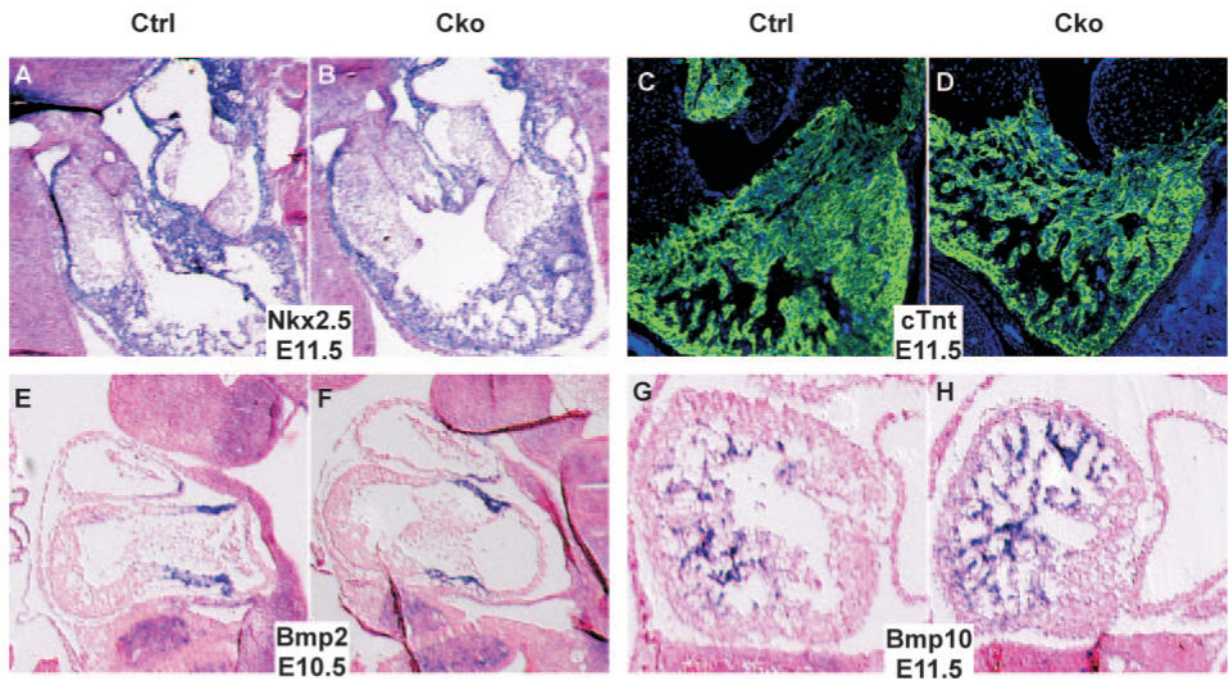


Figure 3.

Molecular examination of mutant embryonic hearts. A and B, Control (A) and mutant (B) embryos at E11.5 were sagittally sectioned. Nonisotope section in situ hybridization analysis was performed with a probe against *Nkx2.5* (purple). Samples were stained with nuclear fast red to visualize nuclei (red). C and D, Immunofluorescence analysis was performed on saggital sections of control (C) and mutant (D) embryos at E11.5 using an antibody against cardiac troponin T (green). Samples were stained with 4',6-diamidino-2-phenylindole to visualize total nuclei (blue). E and F, Section in situ hybridization analysis was performed on saggital sections of control (E) and mutant (F) embryos at E10.5 using a *BMP2* antisense probe (purple). G and H, Section in situ hybridization analysis was performed on saggital sections of control (G) and mutant (H) embryos at E11.5 using probes against *BMP10*. Cko indicates *cTnt-Cre;Smad4^{loxp/loxp}*; Ctrl, *Smad4^{loxp/loxp}*.

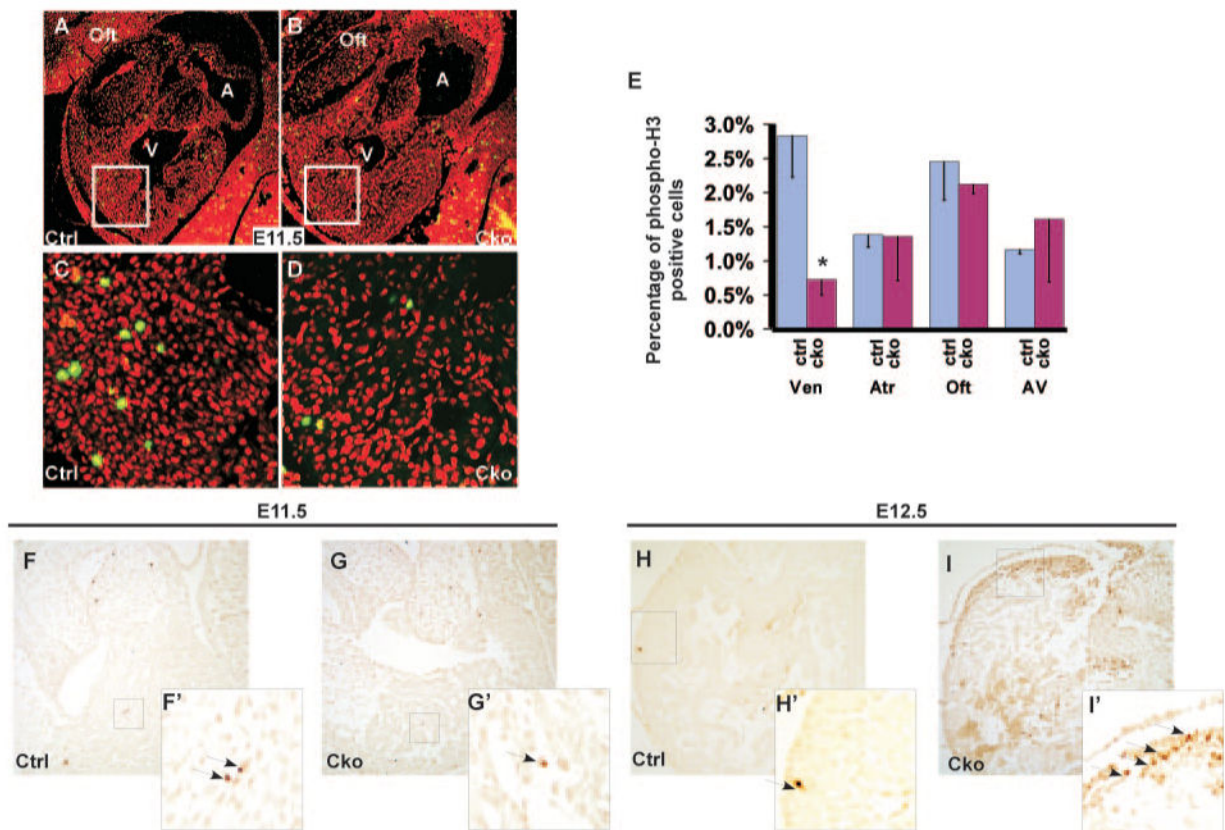


Figure 4.

Myocardial depletion of *Smad4* causes a reduction in cell proliferation and an increase in cell apoptosis. A through E, Sagittal sections of control (A and C) and mutant (B and D) embryos at E11.5 were immunostained with a primary antibody against phospho-H3 (green), and total nuclei were visualized with propidium iodide staining (red). C and D correspond to the boxed regions of A and B, respectively. Quantitative analysis (E) indicates that the proliferation rate in mutant ventricles is significantly reduced compared with the control sample. At least 400 nuclei of each segment of each embryonic heart were counted. Data were averaged from 3 independent embryos with error bars indicating SD. * $P < 0.01$ (Student *t* test). F through I', TUNEL analysis was performed on sections of control (F and F', H and H'), and mutant (G and G', I and I') embryonic hearts at E11.5 and at E12.5. Arrows indicate examples of TUNEL-positive cells. F', G', H', and I' correspond to the boxed regions of F, G, H, and I, respectively. A indicates atrium; Cko, *cTnt-Cre;Smad4^{loxP/loxP}*; Ctrl, *Smad4^{loxP/loxP}*; ofT, outflow tract; V, ventricle.

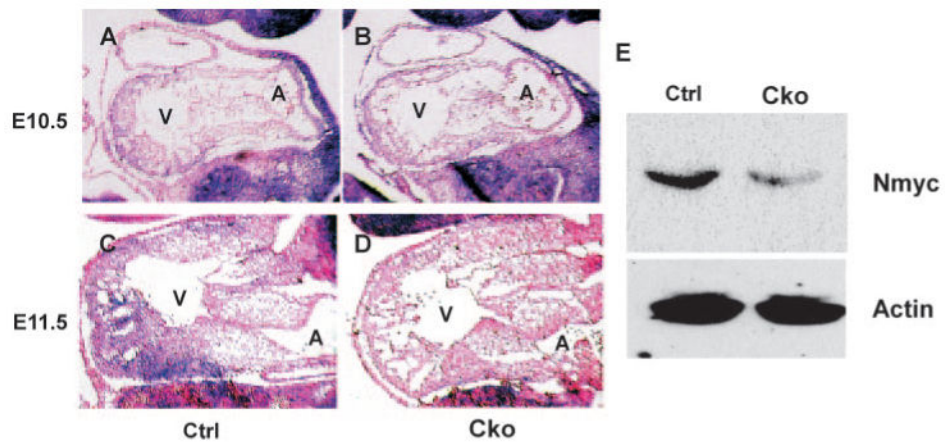


Figure 5.

Expression of *Nmyc* is downregulated by myocardial depletion of *Smad4*. A through D, Section in situ hybridization analysis was performed on sagittal sections of control (A and C) and mutant (B and D) embryos at E10.5 or at E11.5 using an *Nmyc* antisense probe (purple). Samples were counterstained with nuclear fast red (red). E, Proteins were extracted from control and mutant embryonic hearts at E11.5, and Western blot analysis was performed with an anti-*Nmyc* antibody. β -Actin was used as a loading control.

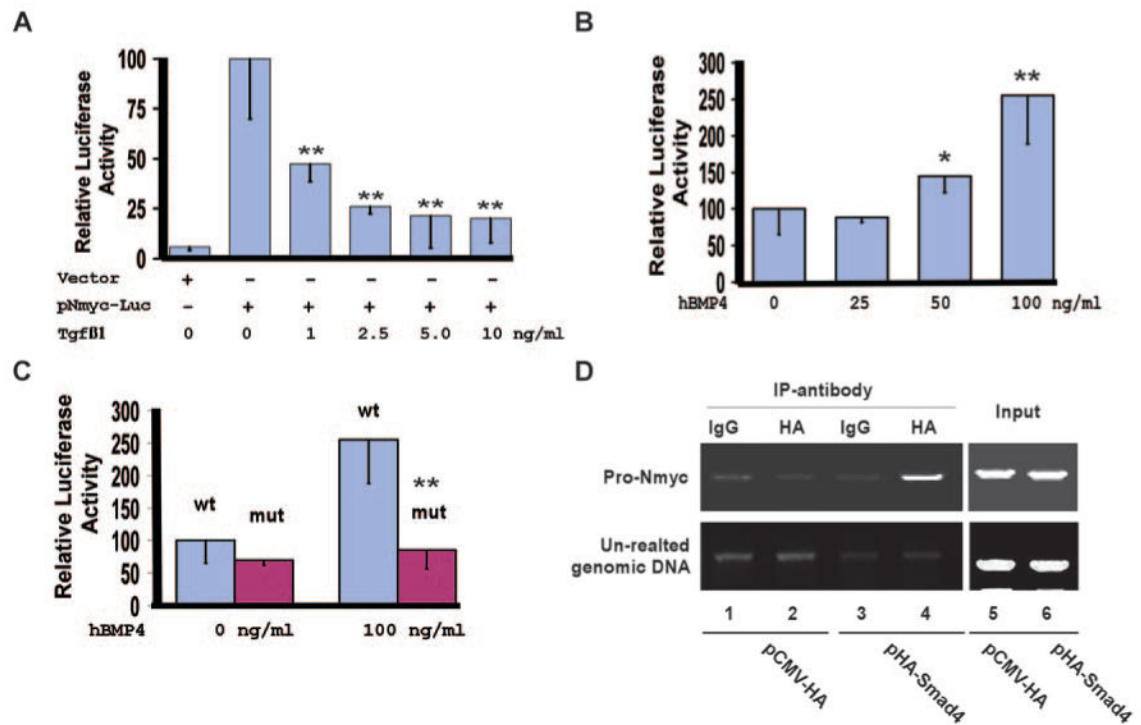


Figure 6.

Nmyc is a direct downstream target of Smad4. A, NIH3T3 cells were transiently transfected with the reporter vector (pGL-basic) or pNmyc-luc, in which expression of luciferase is driven by the 351-bp *Nmyc* promoter. Cells were treated with 0 to 10 ng/mL of TGF β ₁ for 48 hours, and luciferase activities were measured. The luciferase activity of pNmyc-luc without TGF β ₁ stimulation was arbitrarily defined as 100 units. Data were averaged from 3 to 6 independent cultures, with error bars indicating SD. B, P19 cells were transiently transfected with pNmyc-Luc and were treated with 0 to 100 ng/mL hBMP4 for 48 hours. Luciferase analysis was then performed. The activity of the cell cultures without hBMP4 stimulation was defined as 100units. Data were averaged from 6 to 9 independent cultures. C, P19 cells were transfected with pNmyc-luc (wt) or pNmyc-luc-mut (mut), in which the potential Smad4-binding site within the *Nmyc* promoter was disrupted. Cells were treated with 0 or 100 ng/mL of hBMP4 for 48 hours, and luciferase activities were measured. BMP4 failed to upregulate expression of the reporter driven by the mutant *Nmyc* promoter. D, P19 cells were transiently transfected with pCMV-HA vector (negative control) or pHA-Smad4, which expresses HA-Smad4. ChIP analysis was performed with a rabbit IgG or an HA polyclonal antibody. HA-Smad4 formed a complex with the 351-bp *Nmyc* promoter but not with an unrelated genomic DNA sequence. The complex could be captured only by an anti-HA antibody and not by rabbit IgG. * $P < 0.1$, ** $P < 0.05$.

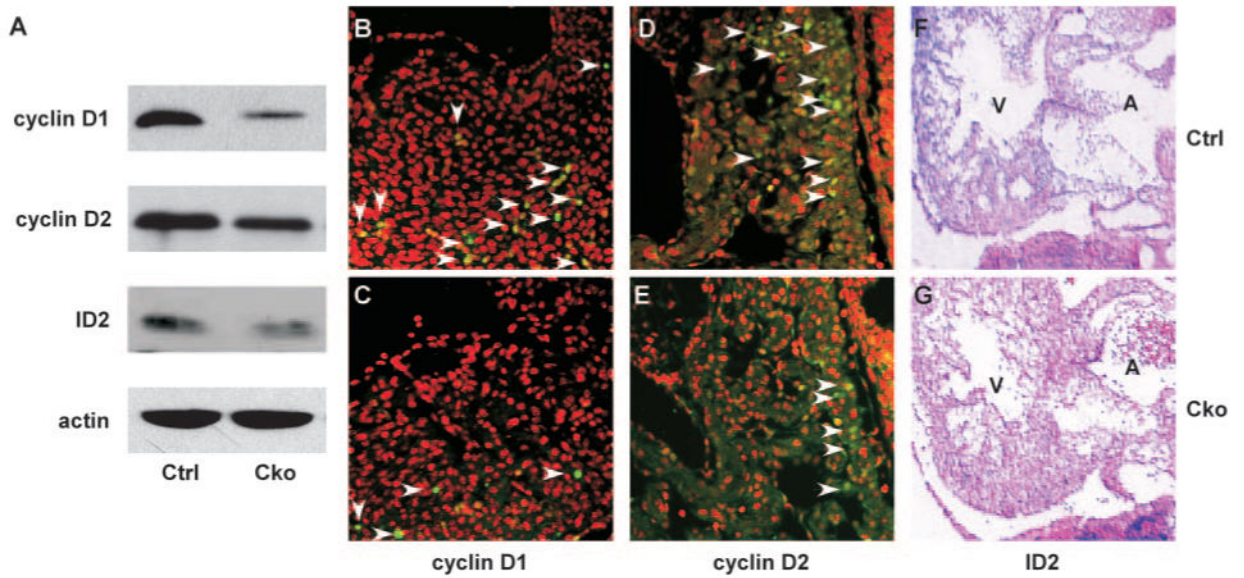


Figure 7.

Downregulation of *Nmyc* target genes in mutant embryonic hearts. A, Proteins were extracted from control and mutant embryonic hearts at E11.5, and Western blot analysis was performed using antibodies against cyclin D1, cyclin D2, and Id2. B through E, Immunofluorescence analysis was performed on sections of control or mutant embryonic hearts at E11.5 using anti-cyclin D1 (B and C) or anti-cyclin D2 (D and E) antibodies (green). Total nuclei were visualized with propidium iodide staining (red). Arrow heads indicate examples of cells with positive signals. F and G, Saggital sections of control and mutant embryos at E11.5 were subjected to section in situ hybridization analysis using an Id2 antisense probe (purple). Samples were counterstained with nuclear fast red (red).

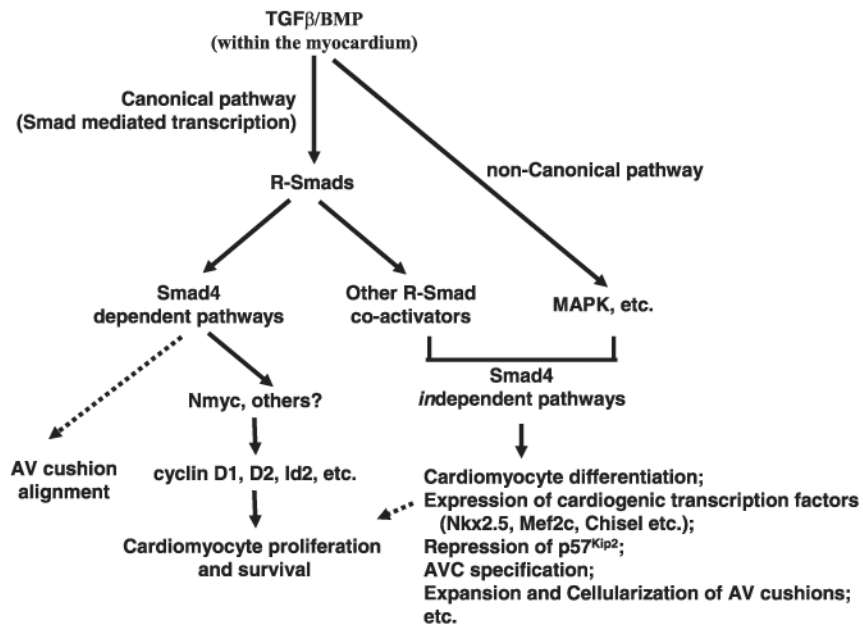


Figure 8. Mediation of TGFβ/BMP signaling through Smad4-dependent and Smad4-independent pathways. Our data showed that *Smad4* is required for proper AV cushion alignment and myocardial cell proliferation and survival. *Nmyc* is a direct target of *Smad4* in the myocardium during embryogenesis.