The Bone Morphogenetic Protein Antagonist Noggin Regulates Mammalian Cardiac Morphogenesis

Murim Choi, Rolf W. Stottmann, Yu-Ping Yang, Erik N. Meyers, John Klingensmith

Abstract—Bone morphogenetic proteins (BMPs) play many roles in mammalian cardiac development. Here we address the functions of Noggin, a dedicated BMP antagonist, in the developing mouse heart. In early cardiac tissues, the Noggin gene is mainly expressed in the myocardial cells of the outflow tract, atrioventricular canal, and future right ventricle. The major heart phenotypes of Noggin mutant embryos are thicker myocardium and larger endocardial cushions. Both defects result from increased cell number. Cell proliferation is increased and cell cycle exit is decreased in the myocardium. Although we find evidence of increased BMP signal transduction in the myocardium and endocardium, we show that the cardiac defects of Noggin mutants are rescued by halving the gene dosage of Bmp4. In culture, BMP increases the epithelial-to-mesenchymal transformation (EMT) of endocardial explant cells. Increased EMT likely accounts for the enlarged atrioventricular cushion. In the outflow tract cushion, we observed an increased contribution of cardiac neural crest cells to the mutant cushion mesenchyme, although many cells of the cushion were not derived from neural crest. Thus the enlarged outflow tract cushion of Noggin mutants likely arises by increased contributions both of endocardial cells that have undergone EMT as well as cells that have migrated from the neural crest. These data indicate that antagonism of BMP signaling by Noggin plays a critical role in ensuring proper levels of cell proliferation and EMT during cardiac morphogenesis in the mouse. (Circ Res. 2007;100:220-228.)

Key Words: BMP signaling ■ endocardial cushion ■ mouse heart development ■ myocardium ■ Noggin

Bone morphogenetic proteins (BMPs) are key regulators of the complex morphogenetic processes in heart development. Explant culture experiments on chick EC tissue demonstrated that BMP is essential in EMT and later events that ultimately give rise to the AV valves. In mouse, conditional gene targeting revealed BMP4 as a myocardial signal that mediates AV and OFT septation defects.

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Ablation of Noggin results in serious developmental abnormalities in multiple tissues, including the neural tube, somites, and skeleton, with perinatal lethality. Because Noggin is expressed in the developing mouse heart, we suspected that cardiovascular abnormalities might also be present in embryos mutant for Noggin. Here we studied the consequences of the absence of Noggin on heart development and demonstrate critical roles for Noggin in EC and myocardial development.

Materials and Methods

Mice
Production and maintenance of Nognull mice, Nognull;Bmp4flox/flox double mutant mice, and Wnt1-Cre;R26R-GFP mice were performed as reported.

Gene Expression Assays, Explant Culture, and Histological Analysis
Histochemistry for β-galactosidase, in situ hybridization, immunohistochemistry, and histology were performed according to standard protocols. For DNA probes and antibodies used, see the online data supplement, available at http://circres.ahajournals.org. Apoptosis was monitored using a terminal deoxy nucleotidyltransferase-mediated dUPT nick-end labeling (TUNEL) assay system, as described. Quantitative measurement of Bmp10 mRNA was performed as described. 3D collagen cultures were prepared as reported, with minor modifications. See the online supplement for details on procedures.

Results

Absence of Noggin Results in Thicker Myocardial Walls and Hyperplastic ECs
To assess whether cardiogenesis is altered in Nognull mutants, we interbred Nognull mice and harvested embryos at various gestational stages. Cardiovascular organs were examined in whole-mount and in histological sections. As reported previously, no Nognull newborn pups were found alive. In the 129S6 and C57BL/6 genetic backgrounds studied here, deletion of Noggin produced heart defects with high penetrance, involving the morphology of myocardial walls, intraventricular septum, and trabeculae of both ventricles (36/38). At E17.5, mutant hearts were pale and smaller, with narrower ventricular lumens (Figure 1A through 1C). Histological examination revealed thickened ventricular walls and intraventricular septum, as well as denser trabeculation (Figure 1D through 1F). Thickened myocardium was also observed in atria (Figure 1G and 1H). Approximately 25% of the mutant hearts also showed membranous ventricular septal defects.

We detected these malformation phenotypes in mutants as early as E11.5, observing both thickened ventricular walls and denser trabeculation (Figure 1I through 1L). To assess whether the number of cardiomyocytes is increased, we assayed expression of MF20, a standard myocardial cell marker. MF20-positive cells were more numerous and multilayered in mutant ventricles, although intracellular myosin structure appeared normal (Figure 1M and 1N). Natriuretic precursor peptide (Nppa) is expressed in both compact and trabecular myocardium in the left ventricle, but right ventricular expression is restricted to trabeculae only. Because of the thicker myocardial wall in the right ventricle, the mutant heart (Figure 1P) showed a broader myocardial domain in which Nppa was absent. This indicates that the expanded tissue is largely compact myocardium.

The second major cardiovascular defect of Nognull hearts was larger ECs. At E11.5, mutant hearts showed enlarged OFT and AV cushions (Figure 2D through 2G and 2H through 2K, respectively; 35/38). To determine whether this enlargement phenotype was caused by increased cell number in the cushions, we counted the number of mesenchymal cells in each cushion. We found a significant increase in both cell number and cushion size in mutants (Figure 2L and 2M). The density of cells in the OFT cushion was also increased, whereas AV cushion cell density remained unchanged (Figure 2N).

We hypothesized that these EC defects might result in perinatal abnormalities, such as OFT septation or AV valve defects. We found several classes of OFT or aortic arch artery patterning defects at birth, such as transposition of great vessels and interrupted aortic arches (data not shown).

Noggin Is Expressed in the Endocardium and Myocardium of the Whole Heart
Several BMPs are expressed in the heart, including the Noggin-binding partners BMP2 and BMP4, in regions relevant to the Nog mutant phenotypes. Bmp2 is expressed in
both the atrioventricular canal (AVC) and OFT myocardium at E9.5; however, the OFT myocardium expression ends by E10.5, whereas strong expression persists in the AVC (Figure 3A). In contrast, Bmp4 expression is restricted to the myocardium of the OFT and is absent from the AVC (Figure 3B). Another member of the BMP signaling family inhibited by Noggin, BMP7 is expressed in all the myocardial domains at all stages assayed (data not shown).

Myocardial Cell Proliferation Is Increased in Embryos Lacking Noggin
Both the thickened myocardial wall and enlarged EC phenotypes were caused by increased cell numbers in the mutant hearts. The underlying defect could involve increased proliferation and/or decreased apoptosis, either of which can be caused by increases in BMP signaling in various contexts.

To compare the expression pattern of Noggin with those of Bmp2 and Bmp4, we stained Nog−/− embryos for β-galactosidase expression. Previous studies confirmed that LacZ expression pattern of Noggin is same as the in situ hybridization pattern. Myocardial expression was detected as early as E8.5, whereas endocardial expression begins at E9.5 (Figure 1 in the online data supplement). Later in development, Noggin is expressed in more defined regions, such as the myocardium of the AVC and OFT at E11.5 and E12.5, although the ventricular expression persists (Figure 3C and 3D and supplemental Figure I). Thus, Noggin shows similar spatiotemporal expression domains to Bmp2 and Bmp4 during formation of the ECs.

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To distinguish between these possibilities, we analyzed levels...
of apoptosis via the TUNEL reaction, and proliferation via anti–phosphorylated histone H3 (pHH3) antibody staining (Figure 4A and 4B). For the cell proliferation assay, we counted pHH3-positive cells and total cell number (visualized by 4′,6-diamidino-2-phenylindole [DAPI] staining of nuclei) and calculated a mitotic index at E10.5 and E11.5 for the whole heart, as well as for the myocardium and endocardium specifically. At both stages, the cell proliferation levels of Noggin mutant hearts were increased (Figure 4C); this was mostly attributable to an increase of myocardial cell proliferation. In contrast, apoptosis appeared comparable to wild-type levels (Figure 4D). Interestingly, cell proliferation and cell death in ECs remained unchanged. Thus, the ventricular myocardium enlargement occurs mainly by increased cell proliferation, whereas the EC enlargement defect must arise by some other mechanism (see below).

To elucidate the basis of increased cardiomyocyte proliferation, we assayed expression of p57kip2, a key negative regulator of cardiac cell cycle exit expressed in myocardium.26,27 At E11.5, Noggin mutant hearts showed reduced expression of p57kip2 in trabecular myocardium as well as in compact myocardium (Figure 4E and 4F). Thus there is decreased expression of a negative regulator of cell cycle exit. Bmp10 is expressed specifically in trabeculated myocardium and negatively regulates p57kip2.26 To test whether Bmp10 expression is also changed by the absence of Noggin, we performed quantitative PCR to measure Bmp10 mRNA from both wild-type and mutant ventricles at E11.5. Bmp10 was expressed at ∼50% higher levels in Noggin mutant hearts (Figure 4G), suggesting that normally Noggin represses Bmp10 expression to control cardiomyocyte proliferation in trabeculated myocardium.

**Higher Rate of Invasive Mesenchymal Cell Formation From Explanted Noggin Mutant Endocardial Cells**

Despite their larger size, the absence of clear changes in proliferation or apoptosis levels in the ECs of Noggin mutant hearts indicates some alternative mechanism underlies their expansion. In addition, the rare mutant hearts exhibiting larger EC tissues, but not thicker myocardium (data not shown), show that these two phenotypes can arise independently. Because cushion formation involves an EMT of endocardial cells, one possibility is that EMT is increased in the mutants.

During EMT, myocardial cells send an initial signal to endocardial cells, which, on receiving the signal, transform into mesenchymal cells and invade the cardiac jelly.3 BMP signaling has been implicated in regulating this process. For example, addition of BMP2 to mouse endocardial explants promoted invasive mesenchymal cell formation in the absence of myocardium; this activity was blocked by the addition of Noggin in the culture medium, indicating that BMP2 is sufficient for EMT in mouse tissue. The expression patterns of Noggin, Bmp2, and Bmp4 in mouse heart tissues are consistent with their being involved in this process (Figure 3). These considerations led us to formulate the following hypothesis: that lack of Noggin in myocardium underlying the cushions causes increased BMP signaling in the myocardium as well as in endocardium, thereby promoting EMT.

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**Figure 4.** Proliferation defect of cardiomyocytes in Noggin mutant hearts. A and B, Immunohistochemistry using pHH3 antibody on wild-type (WT) and mutant hearts shows increased proliferation in Noggin mutant hearts. C, Quantification of staining demonstrates significant increase of positive cells in only mutant myocardial cells in both E10.5 and E11.5 stages. D, No significant change of apoptosis is observed. E and F, Fluorescent-labeled expression of cardiomyocyte-specific cell cycle regulator p57kip2 distribution in wild-type and mutant hearts, showing decrease in the mutant. G, Quantitative PCR of Bmp10 reveals increased expression in Noggin mutant heart. Four embryos from each genotype and stage, with at least 5 sections from each embryo, were compared for statistical analyses. *P<0.02, **P>0.18. Every probability value is >0.22 in D. Scale bars=100 μm.
Increased Neural Crest–Derived Cells in Noggin mutant ECs. A and B, Collagen 3D cultures of E11.5 wild-type (WT) and mutant AV cushions in bright field. C, Higher magnification of B. Arrowheads indicate mesenchymal cells migrated into the gel. D through G, After staining for smooth muscle α-actin (green) and platelet/endothelial cell adhesion molecule (PECAM) (red), cultures were subjected to confocal imaging for optimal visualization. Mesenchyme cells emerged from the aggregates are shown in green. D and E, Wild-type and mutant AV cushion tissue cultures. F and G, OFT cushions. H, Mesenchymal cells were counted after staining (AVC: wild-type=16, mutant=8; OFT cushion: wild-type=10, mutant=9). Boundary of the explant is delineated by a dashed line. In both cases, P<0.002. Using Wnt1-Cre and Rosa26R-GFP strains in a Noggin background, NCCs can be identified by immunostaining for GFP. I and J, Optical sections of wild-type and mutant OFT cushions at E11.5. K and L, Cryosections of E11.5 hearts (K is wild-type and L is Nog−/−). Dashed lines denote OFT cushion boundaries. M, Quantification of neural crest contribution to OFT septum. Scale bars=100 μm.

To explore how BMP signaling might be affecting EC growth in Noggin mutant tissues, we measured the ability of endocardial explants to undergo EMT. OFT cushion and AV cushion tissues were isolated from mouse embryos at E9.5 and E11.5 (Figure 2A) and cultured on collagen gels²¹ for 24 hours (Figure 5A through 5C). For quantification of EMT, we stained the explant with antibodies against smooth muscle α-actin for mesenchymal cell and platelet/endothelial cell adhesion molecule (PECAM) for endocardial cell detection, as described in a previous study.⁶ PECAM-negative actin-positive mesenchymal cells extending out from the explant were counted as mesenchymal cells. To exclude mesenchymal cells that remained on the gel surface (ie, migrated off the explant but did not truly undergo EMT and failed to invade the collagen), we made an optical section just below the explant (Figure 5D through 5G) to allow counting of only the relevant cells.

At E9.5, both OFT and AV cushions showed similar numbers of mesenchymal cells extending out from the wild-type and mutant explants (data not shown). However, mutant tissues from E11.5 embryos showed significantly higher numbers of mesenchymal cells migrating out from both OFT and AV endocardial explants (Figure 5H). These results, together with the unchanged levels of proliferation and apoptosis, strongly suggest that increased EMT levels in Noggin mutant hearts lead to the enlarged ECs.

Increased Neural Crest–Derived Cells in Nog−/− OFT Cushion
Neural crest derivatives are major contributors to the developing OFT cushion.⁹ Because the absence of Noggin increases neural crest production,²⁹ we tested whether incorporation of migrating NCCs might be partially responsible for the larger OFT cushion phenotype. To visualize the neural crest–derived cell population in the cushion, we bred the neural crest–specific Wnt1-Cre driver into the Cre-dependent Rosa26R-GFP reporter line, as described.¹⁰ By examining green fluorescence protein (GFP)-positive cells in wild-type (Wnt1-Cre;R26R-GFP;Nog−/− or Wnt1-Cre;R26R-GFP;Nog+/−) and Nog−/− background (Wnt1-Cre;R26R-GFP;Nog−/−) tissues, we determined the contributions of NCCs in the cushion mesenchyme (Figure 5I through 5L). At E10.5, when cardiac NCCs start to occupy the OFT cushion, their colonization of this tissue was comparable between wild type and Nog−/− (data not shown). However, one day later, an increased contribution of NCCs was clear in mutant cushions (Figure 5I and 5J). Subsequent sectioning and cell quantification confirmed the higher proportion of NCC-derived cells in the Nog−/− cushion (Figure 5K through 5M). This result, along with the previous EMT assay data, suggests that both increased EMT and NCC colonization underlie the enlarged OFT cushion phenotype of Noggin mutants.

Increased BMP Signaling in Nog−/− Endocardial Cells
To assess whether there is altered BMP signaling activity in the cells undergoing EMT in Noggin mutants, we performed immunohistochemistry with anti-phosphorylated Smad1,5,8 antibody, a marker of active domains of BMP signal transduction that has been used in frog, chick, and mouse embryos.³⁰–³² At E9.5, mutant embryos showed increased staining in the caudal pharyngeal arch and OFT regions (Figure 6A and 6B). Transverse sections of these hearts demonstrated increased BMP signaling in Noggin mutant endocardial layers of the OFT (Figure 6C and 6D), as well as of the common ventricle (Figure 6E and 6F).
This demonstrates that lack of Noggin causes increased BMP signaling in endocardial cells during cushion growth stages, when BMP actively promotes the EMT process in these tissues (Figure 5).

**Rescue of Noggin Heart Phenotypes by Reduced BMP4**

Our results strongly suggest that increased BMP signaling in the Noggin mutant heart is responsible for the phenotypes observed. We therefore tested whether reduction of a key BMP ligand could rescue Nog−/− heart defects. As mentioned previously, Noggin antagonizes BMP2 and BMP4 with high efficiency.16 Comparison of expression patterns in the heart raised the possibility that BMP2 and/or BMP4 could be the ligand(s) affected most by the absence of Noggin (Figure 3). We focused on BMP4 because its expression is in the OFT and extends to the myocardium of the right ventricle from an early developmental stage.11

By generating Bmp4+/−;Nog−/− studs and crossing them with Nog+/− females, we compared embryos of the Bmp4+/−;Nog−/− and Bmp4+/−;Nog−/− genotypes as well as other wild-type classes. Dissections were performed during E11.5 to E14.5, and phenotypes were determined by histological sections of each embryo. Consistent with a previous report, removal of one copy of Bmp4 largely rescued spinal neural tube defects in Nog−/− embryos (Figure 7A through 7C).19 Whereas absence of Noggin in the heart showed both larger ECs and thicker myocardial wall phenotypes (Figure 7D and 7E; 12/13), deletion of one copy of Bmp4 in Noggin mutants rescued both heart defects, and the structures were virtually identical to wild type (Figure 7F; 0/14 displayed the mutant phenotypes). These data confirm that the increased BMP signaling we observed in Nog−/− heart tissues underlies the cardiac defects in Noggin mutants and that BMP4 is a key target of the antagonism of Noggin in this context.

**Discussion**

In this study, we have determined the role of the BMP antagonist Noggin in mammalian cardiac development via use of a mouse null mutation of the Noggin locus, in conjunction with embryological assays and molecular expression analysis. Noggin is expressed primarily in the myocar-

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**Figure 6.** Increased BMP signaling in Noggin mutant endocardial cells. Wild-type (WT) and mutant embryos (E9.5) were stained with phospho-Smad1,5,8 antibody for assessing BMP signaling in cells. A and B, Whole-mount view of stained embryos. Brackets indicate caudal pharyngeal arch expression. C and D, Transverse sections of wild-type and mutant heart at the OFT level, exposing both myocardium and endocardium. E and F, Sections of ventricles from wild-type and mutant hearts. Next to each section is a magnified view (C′, D′, E′, F′). Arrowheads indicate increased BMP signaling in endocardium. Scale bars: 100 μm (A and C); 50 μm (C′).

**Figure 7.** Reduction of BMP signaling rescues Noggin cardiac defects. A through C, Dorsal views of each class of embryos at E12.5, indicating the level of section as well as neural tube morphology. D through F, Transverse sections of embryos at the heart level show rescue of Nog−/− cardiac phenotypes by halving gene dosage of Bmp4 (compare E with F; asterisks denote the difference of EC, and brackets indicate the difference in myocardial thickness). Scale bars=0.5 mm.
Noggin Regulates EMT and Neural Crest Migration During Cushion Formation

Our study shows that lack of Noggin results in increased numbers of cells in ECs, causing larger cushions to form. In chick embryos, injection of Noggin-expressing virus into hearts caused underdeveloped ECs and decreased proliferation of cushion mesenchyme cells, in addition to abnormal migration of NCCs into OFT.33 In this case, nonlocalized, high levels of Noggin expression in the heart would be expected to greatly decrease BMP signaling. Consistent with this result, Bmp6;Bmp7 mutant embryos showed an EMT formation defect associated with reduced cell proliferation of myocardial as well as EC cells.13 BMP7 interacts with Noggin, but weakly relative to BMP2 and BMP4.16 No report has yet demonstrated a physical interaction between Noggin and BMP6. Nevertheless, it is tempting to speculate that the exogenous Noggin applied by Allen et al33 titrated and thus reduced functional levels of BMP6 and/or BMP7 to cause a decrease in cushion cell proliferation.

Somewhat surprising was that we detected increased cell proliferation only in myocardial cells and not in endocardial or cushion cells. One explanation for these differences on endocardial proliferation is that the proliferative effects of BMPs antagonized by Noggin on cushion mesenchyme are not categorically proportional to the level of local BMP activity and may disappear when BMP activity is elevated above some threshold. Thus, BMP activity may have a role in ensuring a minimal level of cushion mesenchyme proliferation, but higher levels would not lead to a proportionate increase in proliferation. Alternatively, it is possible that endogenous mouse Noggin does not interact in vivo with BMP7 and BMP6, although we demonstrate that in this context it does interact with BMP4, as predicted from Xenopus studies.16 In this case, absence of increased cushion mesenchyme proliferation in Noggin mutants would reflect a lack of involvement of Noggin in regulating the subset of BMPs controlling this process, as opposed to a major role in regulating those BMPs controlling EMT. Several type I receptors (Alks) for the transforming growth factor-β superfamily are expressed in myocardium and endocardium of the heart. Endothelial-specific deletion of Alk2 generated AV septum and valve defects, from abnormal EMT.34 Furthermore, Alk2 is necessary for the EMT in AV cushion, indicating Alk2 as a critical receptor for BMP signal transduction in cushion formation (Figure 8).

Neural crest contribution is critical for the proper growth of the OFT cushion,8–10 and we observed increased NCCs in the...
enlarged Nog−/− OFT cushion. BMPs have been implicated as a regulator in multiple steps of neural crest development through both Alk2 and Alk3 (Figure 8).9,10,35 Furthermore, a recent study provides evidence that BMPs send a positive signal for generation of neural crest, limited by the BMP antagonists Noggin and Chordin.29 During NCCs migration, Noggin is normally expressed in the dorsal side of pharyngeal endoderm and throughout surrounding mesenchyme (data not shown), which are the locations most likely to affect neural crest migration. Moreover, once NCCs have arrived in the OFT, they may be further affected by increased BMP levels in the heart. Indeed, BMP2/4 in chick heart promotes migration of NCCs into the OFT and maintains the cushion mesenchyme in a proliferative state.33 Based on the expression pattern of Noggin, we believe that Noggin can influence multiple stages of cardiac neural crest development. Once in the OFT, NCCs may be influenced by Noggin from surrounding OFT myocardium (Figure 3). Our results show the consistent roles of BMPs and Noggin on cardiac NCCs and demonstrate a new role of Noggin in cushion development.

**Noggin Regulates Cardiomyocyte Proliferation In Vivo**

Despite extensive studies on the role of BMP signaling in heart development in various model organisms, data addressing the relevance of BMP signaling to mouse myocardial development have been scarce.10,14 Deletion of the BMP receptor type IA gene (Bmpr1a) in a cardiomyocyte-specific manner showed the critical role of BMP1A-mediated BMP signaling in survival of myocardial cells.10 On the other hand, removal of Bmpr1a in the NCC lineage demonstrated a nonautonomous requirement for BMP signaling for normal myocardial cell proliferation.10

Here we demonstrate that removal of Noggin causes increased BMP signaling in the heart. This higher level of BMP signaling in the myocardium showed the opposite effect on cardiomyocytes of the Bmpr1a deletions; namely, increased proliferation. This phenotype was rescued by reduction of Bmp4, indicating that BMP4 is at least one of the relevant BMP ligands antagonized by Noggin in the control of myocardial proliferation. This fits well with the observation that Bmpr1a loss reduces myocardial proliferation, because much evidence suggests that BMP4 signals are transduced by BMP1A (Alk3; Figure 8).36

Although BMP4 is clearly important in the function of Noggin to regulate myocardial proliferation, it is not necessarily the only BMP ligand antagonized by Noggin in this context. This is because multiple BMPs can use the same signal transduction pathway and can be antagonized by Noggin.37 In this regard, it is of interest that Bmp10 null mice displayed a failure of trabecular myocardial formation in association with increased expression of p57kip2 and decreased expression of cardiogenic factors such as Nkx2.5 and Mef2c.28 p57kip2 was identified as a cardiac-specific negative regulator of the cell cycle.26 Although expression levels of cardiogenic markers were normal in Noggin mutants (data not shown), we show reduction of p57kip2 and increase of Bmp10 expression. This result raises the possibility that Bmp10 may be negatively regulated by Noggin. In this scenario, loss of Noggin would increase BMP10 activity and consequently reduce p57kip2 (Figure 8). It would be interesting to test whether the Nog−/−;Bmp10+/− genotype could also rescue the Nog−/− myocardial phenotype.

In summary, Noggin knockout mice exhibited a range of cardiovascular anomalies that resulted from increased BMP signaling, providing more insight into regulation of cardiomyocyte proliferation, endocardial EMT, and neural crest contribution to the OFT cushion, as well as revealing new roles for endogenous Noggin and BMP4 in mouse heart development. On the basis of the morphological similarity of the abnormalities found in Noggin mutants to heart malformations in humans, it is possible that Noggin and BMP may play similar roles in abnormal cardiac morphogenesis in humans. Moreover, understanding the extracellular factors that control embryonic cardiomyocyte proliferation and EMT is of use not only in understanding embryonic developmental mechanisms but also in the eventual design of potential strategies to repair adult cardiac damage.

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**Disclosures**

None.

**References**


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Expanded materials and methods

Mice

All experiments were performed on Nog9E mice on either a 129S6/SvEv inbred or mixed background of 129S6/SvEv and C57BL6/J. This null allele bears an integrated lacZ expression reporter gene. Collection of Nog−/− mutant embryos, timed pregnancies, and genotyping of the mutant (null) and wild-type Noggin allele are described elsewhere.

Production of Nog9E;Bmp4lacZ double mutant mice, originally produced to study spinal development, has also been described. Our production of Wnt1-Cre;R26R-GFP mice, providing a lineage label for neural crest derivatives, was as reported.

Gene expression assays and histological analysis

Whole-mount histochemistry for β-galactosidase activity was carried out as previously described. RNA probes were labeled with digoxigenin (Roche) and used for whole-mount in situ hybridization according to standard protocols. Template plasmid used for in situ hybridization of Nppa is described elsewhere. Immunohistochemistry was performed using MF20 against sarcomeric myofilaments (gift from Margaret Kirby; Developmental Studies Hybridoma Bank), phospho-histone H3 antibody for proliferation assays (Upstate), p57kip2 (Labvision), GFP (gift from Van Bennett) and phospho-Smad1,5,8 antibody (Cell Signaling). MF20 was visualized with Cy3-conjugated antibody (Jackson ImmunoResearch) and other antibodies with Alexa 594 anti-rabbit antibody (Molecular Probes). Apoptosis was monitored using a terminal.
deoxynucleotidyltransferase-mediated dUPT nick end labeling (TUNEL) assay system as described\textsuperscript{2}. Embryos for histological staining were fixed in Bouin’s fix, paraffin-wax embedded and sectioned to 10 $\mu$m. Sections were stained with Hematoxylin and Eosin (H&E) as previously described\textsuperscript{5}. Student’s $t$-test was used for statistical analyses and error bars of graphs equal SEM.

**Endocardial cushion explant culture and analysis**

3-D collagen cultures were prepared as described\textsuperscript{8} with minor modifications. Collagen gel was prepared before embryo dissection and incubated at 37°C for 30 minutes. Endocardial cushion tissues were dissected from the hearts of each wild-type or Nog-- E9.5 or E11.5 embryo and placed on top of the gel, and cultured without liquid media until adding Opti-MEM containing 2% FBS after six hours. After 18 hours of media exposure, the explant was washed with PBS and used for immunostaining using smooth muscle actin (Sigma) and PECAM antibodies. Specimens before and after the immunohistochemistry were photographed using a Leica DFC300 camera and Zeiss LSM 510 confocal microscope, respectively.

**Quantitative RT-PCR (qPCR)**

Quantitative measurement of \textit{Bmp10} mRNA was performed as described\textsuperscript{9}. The following sequences of primers were used for qPCR of \textit{Bmp10}: 5’-TGCCGTCTGCTAACATCATC-3’ (forward); 5’-GTTCCAGCCATGACGACCTCT-3’ (reverse).
References


**Supplemental Figure 1.** Expression of *Noggin* in the developing heart by LacZ staining. (A) Ventral view of *Nog*\(^{+/−}\) heart at E8.5. (B,C) Transverse sections of the OFT regions at E8.5 and E9.5 embryos, respectively. Note the new endocardial expression in C. (D) Ventral view of a slightly older embryonic heart. (E) Section of D, showing expression in compact myocardium as well as trabeculated myocardium. (F) Lateral view showing AVC expression. (G) *Noggin* expression in E11.5 embryonic heart. (H,I) Sections of myocardium of OFT and AV cushion region of the heart shown in G. e, endocardium; m, myocardium; cm, compact myocardium; t, trabeculae; avc, atrioventricular canal; a, atrium; oft, outflow tract. Scale bars in A, G, and K: 100 \(\mu\text{m}\), B and C: 50 \(\mu\text{m}\).