# An Nkx2-5/Bmp2/Smad1 Negative Feedback Loop Controls Heart Progenitor Specification and Proliferation

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DOI 10.1016/j.cell.2007.01.042

## SUMMARY

During heart development the second heart field (SHF) provides progenitor cells for most cardiomyocytes and expresses the homeodomain factor Nkx2-5. We now show that feedback repression of Bmp2/Smad1 signaling by Nkx2-5 critically regulates SHF proliferation and outflow tract (OFT) morphology. In the cardiac fields of *Nkx2-5* mutants, genes controlling cardiac specification (including *Bmp2*) and maintenance of the progenitor state were upregulated, leading initially to progenitor overspecification, but subsequently to failed SHF proliferation and OFT truncation. In *Smad1* mutants, SHF proliferation and deployment to the OFT were increased, while *Smad1* deletion in *Nkx2-5* mutants rescued SHF proliferation and OFT development. In *Nkx2-5* hypomorphic mice, which recapitulate human congenital heart disease (CHD), OFT anomalies were also rescued by *Smad1* deletion. Our findings demonstrate that Nkx2-5 orchestrates the transition between periods of cardiac induction, progenitor proliferation, and OFT morphogenesis via a Smad1-dependent negative feedback loop, which may be a frequent molecular target in CHD.

## INTRODUCTION

The logic of molecular pathways underpinning heart development is poorly understood, and little is known about how such pathways are perturbed in congenital heart disease (CHD). Myocytes of the early heart tube derive from at least two distinct cardiac progenitor cell populations. The cardiac crescent and primary heart tube are derived from cells of the first heart field (FHF) that begin differentiation at the crescent stage. The second heart field (SHF) is a population of undifferentiated multipotent cardiac progenitor cells that proliferate and contribute dynamically to heart tube growth at both inflow and outflow poles, providing the majority of myocytes for the right ventricle (RV) and outflow tract (OFT), as well as contributions to the left ventricle (LV) and atria (Buckingham et al., 2005).

A number of factors have been implicated in the development of SHF progenitors, including transcription factors Tbx1, Islet-1 (Isl1), Foxh1, Mef2c, and Hand2, and fibroblast growth factors Fgf8 and Fgf10 (Buckingham et al., 2005). These genes are expressed in SHF cells, and most are downregulated upon SHF cell differentiation, suggesting progenitor-specific roles (Kelly, 2005). The T-box transcription factor Tbx1 is a positive regulator of SHF proliferation (Xu et al., 2004), potentially via direct control of genes for fibroblast growth factors Fgf8 and Fgf10 (Hu et al., 2004; Vitelli et al., 2002; Xu et al., 2004). Isl1, along with Gata4 and Foxh1, controls expression of *Mef2c*, a transcription factor essential for specification of both cardiomyocytes and endothelial lineages (Dodou et al., 2004; von Both et al., 2004).

The homeodomain factor Nkx2-5 sits high in the cardiac regulatory hierarchy and is expressed in cells of both the FHF and SHF (Stanley et al., 2002). The human gene, *NKX2-5,* is to date the most commonly mutated single gene in CHD, accounting for 1%–4% of specific malformations, including disruption of the interatrial wall (atrial septal defect; ASD), malpositioning of the outflow vessels

(double-outlet right ventricle; DORV), and a complex congenital condition arising from stenosis of the pulmonary artery termed tetralogy of Fallot (Benson et al., 1999; Elliott et al., 2003; McElhinney et al., 2003; Schott et al., 1998). Studies in mice show that *Nkx2-5* is required for specification and spatial definition of chamber myocardium and for formation and maintenance of elements of the conduction system (Habets et al., 2002; Jay et al., 2004; Lyons et al., 1995; Pashmforoush et al., 2004; Tanaka et al., 1999). However, expression of *Nkx2-5* in cardiac progenitor cells suggests uncharacterized roles for this gene at the earliest stages of cardiogenesis.

Here we use microarray to compare the transcriptomes of *Nkx2-5* heterozygous and null mutant embryos and identify an important early role for Nkx2-5 as a negative regulator of genes responsible for cardiac induction and the progenitor state. Furthermore, we provide evidence using *Nkx2-5* hypomorphic mice that an Nkx2-5/Bmp2/Smad1 negative feedback pathway is a molecular target in CHD.

## RESULTS

To map progenitor cell descendants in *Nkx2-5* mutant hearts, we examined expression of region-specific transgenes. The *Mlc3f-nlacZ-2E* transgene normally marks the LV and right atrium in the looping heart (Figure 1A) (Kelly et al., 1997), and expression in the single ventricle-like chamber of *Nkx2-5* null embryos (Figure 1B) suggests that this chamber is a correlate of the normal LV and mainly derived from the FHF. The narrow, short OFT in mutants was negative for the transgene.

To explore the progenitor composition of mutants further, we examined the expression of transgenes *Mlc2v-lacZ*, *Mef2c-lacZ*, and *Pitx2c-lacZ* that mark the OFT and RV, derivatives of the anterior SHF (Dodou et al., 2004; Kelly et al., 2001; Ross et al., 1996; Shiratori et al., 2001). All transgenes were expressed in the short outflow region of *Nkx2-5* mutant hearts (Figures 1C–1H), showing that this region contains SHF descendants. The *Mlc2v-lacZ* transgene additionally marks a population of SHF-derived cells at the outflow pole of the LV (Cai et al., 2003; Ross et al., 1996; Verzi et al., 2005), and expression was evident at the outflow pole of the ventricle-like chamber in *Nkx2-5* mutants (Figures 1C and 1D). This LV SHF contribution was not highlighted by expression of *Mef2c-lacZ* (Figures 1E and 1F), as this transgene is known to be silenced in LV cells (Verzi et al., 2005). *Pitx2c-lacZ* is expressed in descendants of the left anterior SHF that have been exposed to the left/right morphogen nodal (Shiratori et al., 2001), and reduced expression in the OFT of null embryos (Figures 1G and 1H) confirmed that the SHF contribution to the heart tube was compromised. Explants from the SHF region of both wild-type and mutant embryos (Zaffran et al., 2004) expressed *Mlc1v-nlacZ-24*, marking the SHF (Kelly et al., 2001) but not the LV-specific *Mlc3f-nlacZ-2E* transgene, and this signature was stable after 48 hr of in vitro culture (data
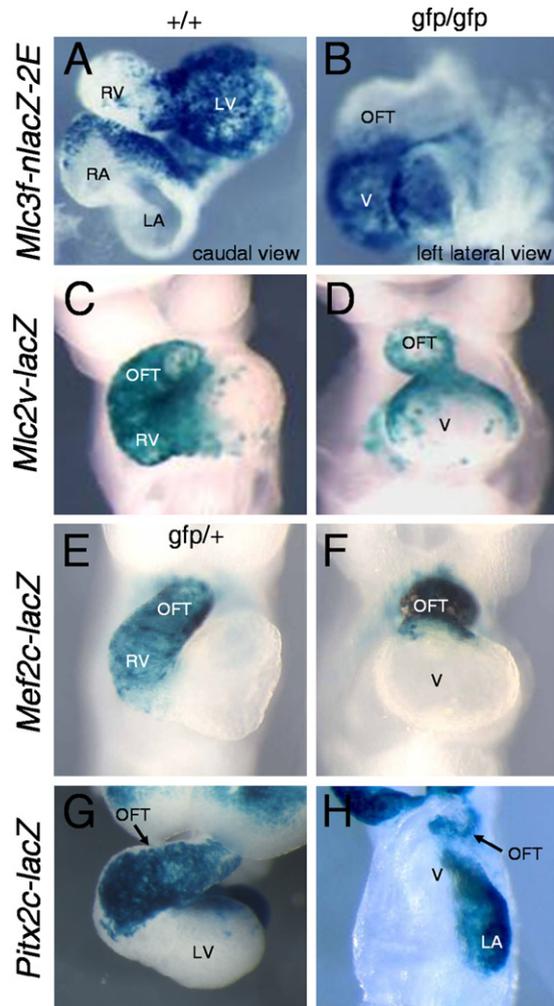


**Figure 1. Analysis of FHF and SHF Contributions to *Nkx2-5*-Null Embryos**

(A–H) Expression of indicated lacZ transgenes in wild-type (+/+) and *Nkx2-5*^gfp/gfp^-null (gfp/gfp) embryos at E9.0. The following abbreviations are used: OFT, outflow tract; LV, left ventricle; LA, left atrium; RA, right atrium; RV, right ventricle; V, ventricle-like chamber in null embryos.

not shown). These data show that both the FHF and SHF contribute to *Nkx2-5*-null hearts—a primitive LV is established relatively normally from FHF progenitors (albeit with some delay), but the grossly truncated OFT and indistinct RV highlight a prominent SHF defect.

### Microarray Profiling of *Nkx2-5*-Null Hearts

To identify genes dysregulated in the heart and heart fields of *Nkx2-5* mutants, cDNA microarray was performed using cells purified by fluorescence-activated cell sorting (FACS) from embryos carrying an *Nkx2-5*^gfp^ knockin allele that expresses enhanced green fluorescent protein (GFP) under *Nkx2-5* control (Biben et al., 2000) (Figure 2A). Sorted cells from single somite-matched embryo pairs (*Nkx2-5*^gfp/+^ versus *Nkx2-5*^gfp/gfp^) were compared at nine
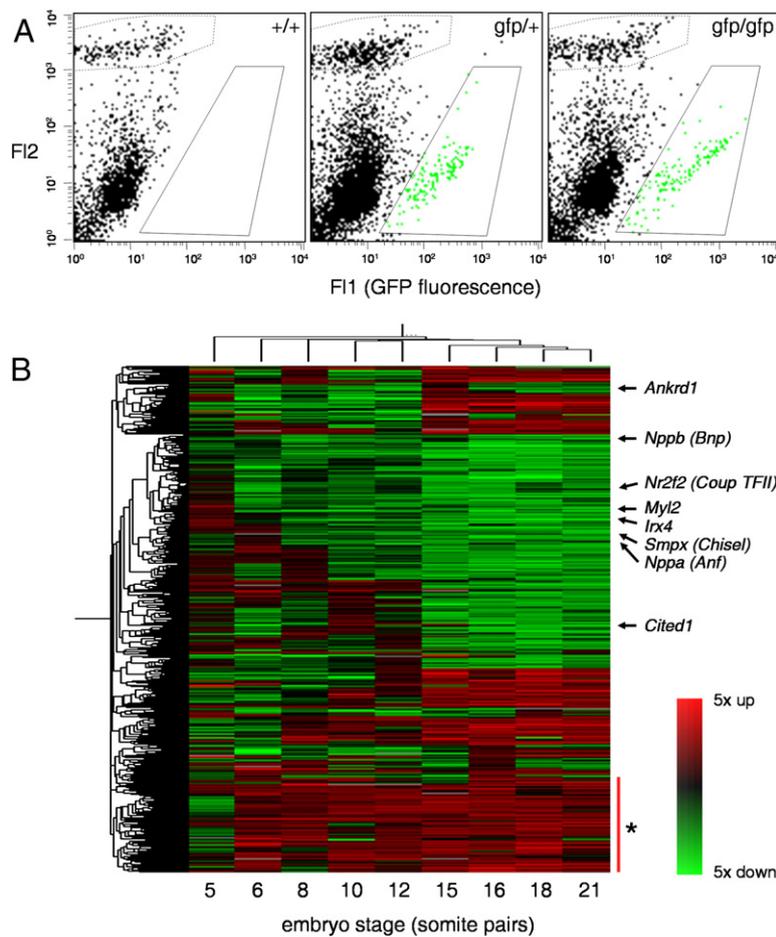
**Figure 2. Microarray Analysis**

(A) Fluorescence channels 1 (Fl1, 530 nM) and 2 (Fl2, 585 nM) FACS plots of wild-type (+/+), *Nkx2-5*[gfp/+] heterozygous, and *Nkx2-5*[gfp/gfp]-null embryos (both axes $\log_{10}$ scale). Gates define propidium iodide-permeable dead cells (dashed line) and live *Nkx2-5*-GFP^{+} cells (solid line). *Nkx2-5*-GFP^{+} cells (green) were used for microarray analysis.

(B) Microarray heat map showing fold change in mRNA expression between *Nkx2-5*-null and heterozygous GFP+ cells (see scale). Genes modified at least 1.7-fold at three embryonic stages were clustered by standard correlation analysis (see tree diagrams for genes, left, and for embryonic stages, above). Genes known to be downregulated in *Nkx2-5* mutants are arrowed. The prominent cluster of genes upregulated from 6 to 21 ps is indicated (*).

different stages from E8.0 (5 pairs of somites [ps]) to E9.5 (21 ps). Genes with at least a 1.7-fold change in three or more stages were selected (923 of ∼16 K unique genes). Unsupervised hierarchal clustering (Eisen et al., 1998) correctly ordered all embryonic stages, and known *Nkx2-5*-dependent genes were identified (Figure 2B). Myofilament protein genes were not selected, confirming that cardiomyocyte differentiation is unaffected in mutants (Lyons et al., 1995).

**Nkx2-5 Negatively Regulates Cardiac Progenitor Genes**

The most numerous and substantial gene-expression changes were from 15 to 21 ps, with functional annotation suggesting a preterminal state (see Figure S1 legend). We assessed earlier stages and saw upregulation of a cohort of genes from 6 to 21 ps in mutants (Figure 2B). Twenty genes expressed at 8–12 ps were prioritized using a < 1/100 false-positive cut-off (Tusher et al., 2001), and their upregulation was confirmed by quantitative (q) RT-PCR (Figure S1A) and in situ hybridization (ISH). Region-specific expression was observed for 9 out of 20 upregulated genes by ISH. All nine were expressed in cardiac progenitor cells, i.e., precardiac mesoderm and the early cres-

cent, with all bar one (*Tbx5*) downregulated in the forming heart tube. Seven genes were previously unrecognized markers for cardiac progenitors.

All were expressed, albeit transiently, in the precardiac region and early cardiac crescent, examples being genes for insulin-like growth factor binding protein (Igfbp5), platelet-derived growth factor receptor α (Pdgfra), and transmembrane protein Odz4 (Figures 3A, 3B, 3E, 3F, and S2A–S2C). Later patterns identified novel subpopulations within the SHF, e.g., at late crescent stages, genes for Igfbp5, Pdgfra, and Odz4, as well as matrix protein tenascin C (Tnc) and homeodomain transcription factor Pbx3 were expressed most strongly in caudal SHF progenitors (Figures 3A, 3B, 3E, 3F, 3M, 3N, and S2A–S2D). Anteriorly, *Tnc* was expressed in the lateral reaches of SHF mesoderm (wild-type embryos in Figures 3M–3O), while *Pdgfra* was expressed more medially in dorsal mesocardium.

In *Nkx2-5*-null embryos, most of these progenitor genes failed to undergo downregulation in definitive cardiac structures; e.g., at E8–8.5 *Igfbp5* and *Pdgfra* expression persisted throughout the entire early heart tube (Figures 3A–3H). We also examined expression of Pdgfra protein. In wild-types, expression was seen in the cardiac
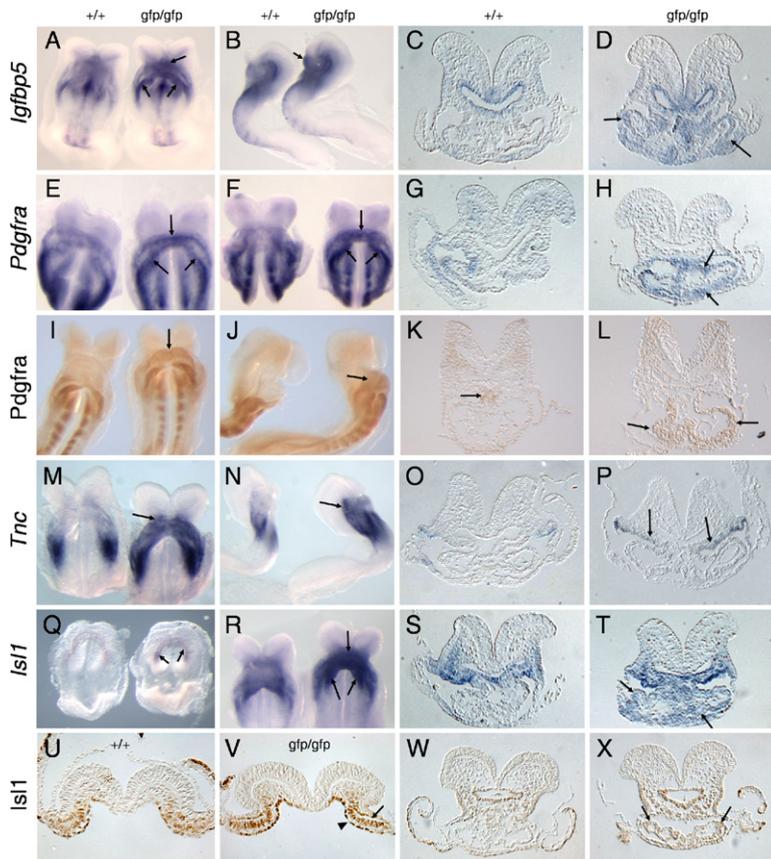
**Figure 3. Cardiac Progenitor Genes/Proteins Upregulated in *Nkx2-5*-Null Embryos**

(A–X) Whole mount ISH and immunohistochemistry with representative sections showing expression of indicated cardiac progenitor cell genes/proteins in wild-type (+/+) and *Nkx2-5^gfp/gfp^* embryos at E7.5-E8.5. Regions of upregulated expression in *Nkx2-5* mutants are indicated (arrows, cardiac mesoderm; arrowheads, endoderm).

crescent then strongly in caudal progenitors and dorsal mesocardium (Figures 3I and 3K), resembling the mRNA pattern (Figures 3E–3G). In null embryos, expression was seen throughout the heart tube (Figures 3J and 3L). Expression of *Tnc* did not persist in the mutant heart tube but was strikingly upregulated in the dorsal mesocardium and dorsal pericardial SHF mesoderm (Figures 3M–3P).

### Isl1 Is a Pan-Cardiac Progenitor Marker Negatively Regulated by *Nkx2-5*

We examined expression of SHF marker *Isl1*, not present on our arrays. In wild-type embryos *Isl1* transcripts were abundant in the SHF but undetectable in cardiomyocytes of the crescent and heart tube (Figures 3Q–3S), supporting published data (Cai et al., 2003). However, in *Nkx2-5*-null embryos, *Isl1* expression persisted in cardiomyocytes of the cardiac crescent and heart tube (Figures 3Q–3T). By qRT-PCR, *Isl1* mRNA was upregulated 4-fold in FACS-purified GFP+ cells from *Nkx2-5^gfp/gfp^* embryos at 6–12 ps (Figure S1A).

In contrast to *Isl1* mRNA, Isl1 protein was expressed in E7.5 wild-type embryos throughout the anterior intraembryonic coelomic walls and proximal head mesenchyme, regions that encompass both the FHF and SHF (Figure 3U), as well as in ventral and lateral foregut endoderm. Thus, Isl1 is likely to be a pan-cardiac progenitor marker in mouse. By E8.0, Isl1 expression was lost in differentiating cardiomyocytes of the late crescent/forming heart tube (Figure 3W). In *Nkx2-5*-null embryos, however, Isl1 levels were increased in anterior coelomic walls and foregut endoderm at E7.5, and Isl1 persisted in cardiomyocytes of the late cardiac crescent and heart tube (Figures 3V and 3X). Expression of *Mef2c* and *Tgfβ2*, other SHF genes not present on the array, also persisted in the heart tube of *Nkx2-5* mutants (Figures S2E and S2F).

### Quantitative Analysis of Cardiac Progenitor Phenotype in Nkx2-5-Mutant Embryos

We used the novel cardiac progenitor marker Pdgfra to perform a quantitative FACS analysis of the persistent progenitor phenotype seen in Nkx2-5-null hearts. Nkx2-5-null embryos harbored a ~2-fold increase in the number of Nkx2-5-GFP+/Pdgfra+ progenitor cells from 6 to 20 ps (Figure S3A), attributable to the persistent expression of Pdgfra in the mutant heart tube (Figures 3E–3L). Importantly, mean Pdgfra expression per cell was not altered in Nkx2-5-null embryos (not shown). Other features of this quantitative analysis were noteworthy. In Nkx2-5^gfp/+^ controls, both cranial and caudal Nkx2-5-GFP+ SHF subpopulations were characterized by ~5-fold lower mean levels of Nkx2-5-GFP compared to differentiating myocytes (Figure S3B). Furthermore, mean GFP expression per cell was higher in both Nkx2-5-GFP+ and
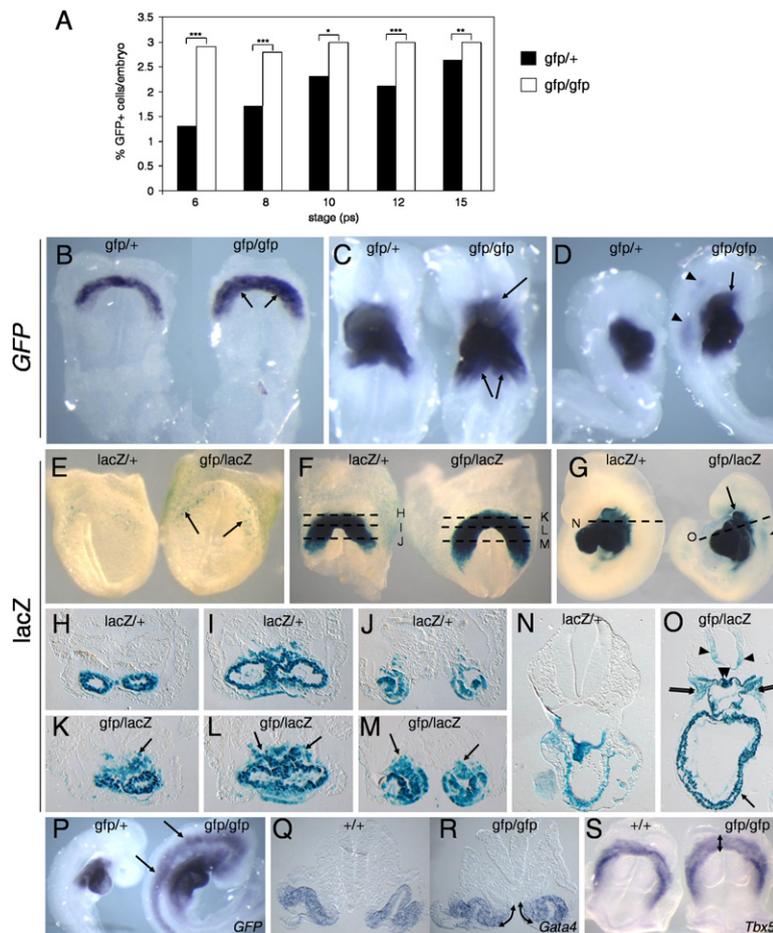
**Figure 4. Overspecification of Cardiac Progenitors in *Nkx2-5*-Null Embryos**

(A) Percentage of *Nkx2-5*-GFP^{+} cells/embryo in *Nkx2-5^(gfp/+)* control and *Nkx2-5^(gfp/gfp)*-null embryos detected by FACS (6–15 ps). An average of ~7200 cells (6–10 ps) and 23,000 cells (12–15 ps) were counted for each sample. Asterisks show significance by chi-square analysis (***p < 0.001, **p < 0.01, *p < 0.05).

(B–D) *GFP* mRNA expression in *Nkx2-5^(gfp/+)* and *Nkx2-5^(gfp/gfp)*-null embryos at E7.75 (B) and E8.5 (C and D). Ectopic *Nkx2-5-GFP* expression is indicated in tissues adjacent to the heart (arrows) and in neural tube (arrowheads).

(E–O) X-gal staining for *Nkx2-5*-lacZ expression in *Nkx2-5^(lacZ/+)* control and *Nkx2-5^(gfp/lacZ)*-null embryos at E7.5 (E), E8.0 (F and H–M) and E9.5 (G,N, and O). Ectopic *Nkx2-5*-lacZ expression in the cardiac region (arrows), pharyngeal mesenchyme (double arrows), neural tube (arrowheads), and foregut (double arrowheads) is indicated. Dashed lines in (F) and (G) indicate planes of sections in H–M and N and O, respectively.

(P) *GFP* mRNA expression at E9.0 in *Nkx2-5^(gfp/+)* and *Nkx2-5^(gfp/gfp)* embryos showing induction in neural tube and head mesoderm remote from the cardiac region in mutants.

(Q and R) Expanded expression of *Gata4* at E8.0 in the medial wall of the intraembryonic coelom (arrows) in *Nkx2-5^(gfp/gfp)* embryos.

(S) Expanded expression of *Tbx5* at E7.75 in *Nkx2-5^(gfp/gfp)* embryos (arrow).

Nkx2-5-GFP[+]/Pdgfra^{+} cells isolated from mutants compared to heterozygotes (10–20 ps), suggesting that, as for other progenitor genes, Nkx2-5 is a target of negative feedback regulation (Figures S3B–S3D).

### *Nkx2-5* Limits Cardiomyocyte Specification

The data above show that *Nkx2-5* is essential for repressing early cardiac progenitor genes. We explored whether this repressive activity served to limit cardiac specification. Assessed by FACS, the number of *Nkx2-5*-GFP^{+} cells was greater in mutants than in heterozygous controls from 6 to 15 ps (Figure 4A). The effect was more pronounced at earlier stages, with ~2-fold more *Nkx2-5*-GFP^{+} cells in mutants at 6–8 ps. ISH using a *GFP* probe suggested expansion of *Nkx2-5* expression specifically in SHF cells, i.e., medial to the cardiac crescent at E7.75 and dorsolateral and anterior to the heart tube at E8.5 (Figures 4B–4D).

We confirmed an expansion in the number of *Nkx2-5*-expressing cells using lacZ staining of *Nkx2-5^(lacZ/+)* heterozygous and *Nkx2-5^(gfp/lacZ)*-null embryos, matched for dosage of an *Nkx2-5-lacZ* knockin allele (Elliott et al., 2006). In null embryos at E8.0 (5 ps), lacZ was more broadly expressed in mesoderm dorsal to the heart tube at all ante-

rior-posterior levels (Figures 4F and 4H–4M). This finding was not biased by the negative autoregulatory action of *Nkx2-5* that became evident only after 10 ps (Figure S3B). We examined lacZ expression at the earliest time of its onset in anterior lateral plate mesoderm at E7.5 (Figure 4E). Even at this stage, expression was evident in more cells in null embryos. Expanded expression of early cardiac transcription factor markers, *Gata4* and *Tbx5*, in null embryos at E7.75–8.0 (Figures 4Q–4S) confirmed that there was an expansion of the FHF at early stages in mutants. *Tbx5* was upregulated 2-fold in null embryos at E8.0–8.5 by microarray.

### Elevated Bmp2 Signaling in *Nkx2-5*-Null Embryos

*Nkx2-5* was also ectopically expressed in the ventral neural tube from ~E8.75, as detected by ISH for *GFP* and lacZ staining in respective mutant strains, an effect that increased dramatically with further development (Figures 4D, 4G, 4N–4P, and S2G). Neural *Nkx2-5* expression was not secondary to a preterminal state because it was absent in similarly compromised *Tbx20^(lacZ/lacZ)*-null embryos (Figure S2H) (Stennard et al., 2005). This suggested the action of a long-range diffusible morphogen accompanying the increased specification of cardiac progenitors
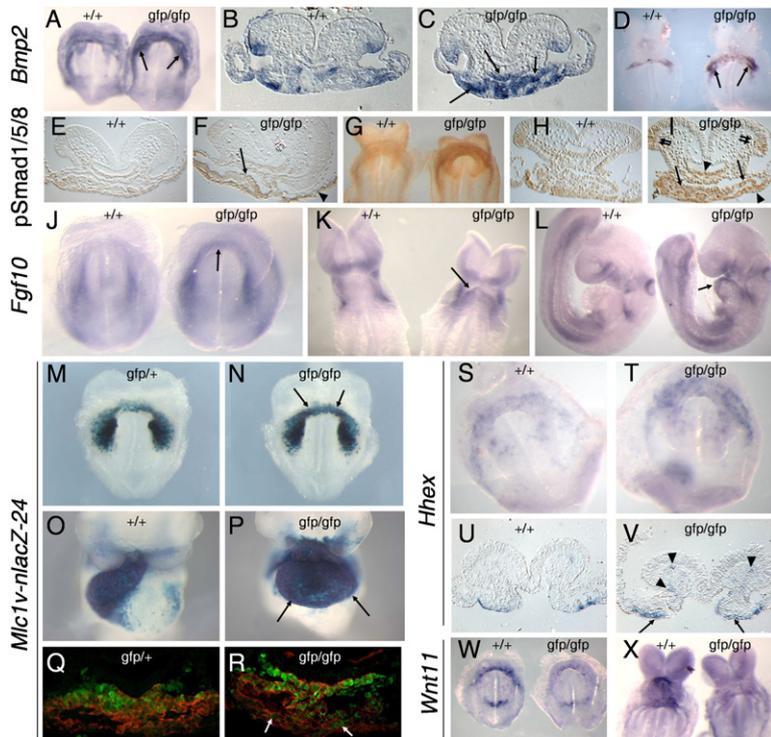
**Figure 5. Modified Expression of Cardiac Signaling Components in *Nkx2-5*-Null Embryos**

(A–D) *Bmp2* expression in wild-type (+/+) and *Nkx2-5^gfp/gfp*-null embryos at E8.0 (A–C) and E8.5 (D). Elevated expression in mutants arrowed.

(E–I) Phospho-Smad1/5/8 expression at E7.75 (E and F) and E8.0 (G–I). Elevated expression in mutants are indicated in cardiac mesoderm (single arrows), endoderm (arrowheads), and ventral neural tube (double arrows).

(J–L) *Fgf10* expression at E7.5 (J), E8.5 (K), and E9.5 (L), arrows showing ectopic expression.

(M–P) β-galactosidase activity from the *Mlc1v-nlacZ-24* transgene at E8.0 (M and N) and E9.5 (O and P), with arrows showing ectopic expression.

(Q and R) Coimmunodetection of β-galactosidase protein from the *Mlc1v-nlacZ-24* transgene (green nuclei) and sarcomeric myosin (red) at E7.5 in the cardiac crescent in *Nkx2-5^gfp/+* control and *Nkx2-5^gfp/gfp* embryos. Arrows indicate expanded expression of β-galactosidase in myosin+ cardiomyocytes in mutants. (S–V) *Hhex* expression at E7.75 showing elevated expression in cardiac endoderm (arrows) and endothelial cells within cranial mesenchyme (arrowheads) of *Nkx2-5^gfp/gfp* embryos.

(W and X) *Wnt11* expression (arrows) is severely reduced in *Nkx-2-5*-null embryos at E7.75 (Y) and E8.5 (Z).

in *Nkx2-5*-null embryos. We therefore examined the expression of known secreted cardiac-inducing factors. *Bmp2* and *Fgf10* mRNAs were elevated in mutant FACS-purified *Nkx2-5*-GFP+ cells by microarray and qRT-PCR (3- to 6-fold and 2- to 15-fold, respectively; Figures S1A and S1B). By ISH, *Bmp2* mRNA was elevated in mutants in the cardiac crescent (E8.0; Figures 5A–5C) and then at the pericardial/myocardial boundaries at the poles of the heart tube (E8.5; Figure 5D). *Bmp4* and *Bmp7* were not upregulated. Bmp signaling through Smad1/5/8 phosphorylation (pSmad1/5/8) was also increased in mutant precardiac mesoderm (E7.75; Figures 5E and 5F), then in the fusing heart tube and SHF (E8.0; Figures 5G–5I). Elevated phospho-Smad1/5/8 extended to anterior foregut and visceral endoderm of null mutants (Figures 5E–5I) as well as to neural ectoderm from E8.0–9.5 (Figures 5H and 5I). While pSmad was elevated broadly in mutants, *Bmp2* upregulation remained confined to its normal pattern of expression, indicating an enhanced paracrine and indeed long-range effect of mesoderm-expressed Bmp2 on endoderm and ectoderm. Expression of genes encoding Bmp inhibitors, Cer1, Dan, and noggin was not altered in *Nkx2-5* mutants (not shown).

*Fgf10* was also upregulated and more broadly expressed in the cardiac crescent region of *Nkx2-5* mutants at E7.5, as seen by ISH (Figure 5J), and expression persisted throughout the mutant heart tube (E8.5–9.5; Figures 5K and 5L). *Fgf8* expression was not elevated

(Figure S1B). The *Mlc1v-nlacZ-24* transgene traps an *Fgf10* enhancer expressed in the SHF and in its anterior derivatives, the OFT/RV (Kelly et al., 2001). In mutants, increased β-galactosidase activity was evident in cardiomyocytes of the crescent, then whole heart tube at E9.5 (Figures 5M–5P), similar to findings for *Fgf10* mRNA. Double immunostaining of sections from *Mlc1v-nlacZ-24* embryos at the late crescent stage showed some overlap between myosin expression, marking the zone of differentiation in FHF and SHF cells, and lacZ expression, which marks the SHF. In *Nkx2-5* mutants, there was greater encroachment of β-galactosidase protein expression into the FHF in the cranial region (E7.75; Figures 5Q and 5R), indicating a very early effect on *Fgf10*.

The homeodomain repressor *Hhex* is expressed in anterior visceral and foregut endoderm and, when overexpressed in frog blastomeres, can induce *Nkx2-5* expression in adjacent mesoderm (Foley and Mercola, 2005). *Hhex* was upregulated in *Nkx2-5* mutants in anterior endoderm underlying the cardiac crescent (Figures 5S–5V) as well as in endothelial progenitors within head mesenchyme, again suggestive of the action of a long-range morphogen. *Hhex* may be induced in mutants by upregulated Bmp2 via its Smad-responsive enhancer (Zhang et al., 2002). Expression of *Dkk1*, encoding a canonical Wnt inhibitor that can also induce *Hhex* (Foley and Mercola, 2005), was unaffected. Cardiogenesis can be stimulated in frog embryos by the noncanonical Wnt

pathway ligand, Wnt11 (Pandur et al., 2002). However, *Wnt11* was downregulated in mutants (Figures 5W, 5X, 6O, and 6P).

### *Nkx2-5* Is Required for SHF Proliferation through Suppression of Bmp2/Smad1 Signaling

Impaired proliferation or deployment of SHF cells may underpin the dramatically narrowed and shortened OFT seen in *Nkx2-5* mutants. We examined proliferation of SHF progenitors in mutants, scoring for phosphohistone H3. As reported (Tanaka et al., 1999, 2001), foregut endoderm proliferation showed a reduction by 44% in mutants (p = 0.011; Figure 6A), but cardiomyocytes were unaffected. However, we also found that proliferation was strikingly reduced in dorsal pericardial SHF cells (80% reduced, p < 0.0001) in the absence of effects on cell death (not shown).

The Bmp inhibitor noggin stimulates cell proliferation in chick SHF explants (Waldo et al., 2001). To examine whether Bmp/Smad signaling represses SHF progenitor proliferation in vivo, we deleted loxP-flanked (floxed; fl) *Smad1* alleles (Tremblay et al., 2001) specifically in anterior mesoderm, using *Mesp1-Cre* (Saga et al., 1999). Strikingly, *Mesp$^{Cre/+}$/Smad1$^{fl/fl}$* embryos showed a 2.3-fold increase in proliferation of SHF cells at E8.5 (p < 0.0005; Figure 6B) but no change in myocyte proliferation. Proliferation was unaffected in foregut endoderm (Figure 6B) that expressed Smad1 (Figure 5H) but not *Mesp1-Cre* (Saga et al., 1999), and we conclude, therefore, that the observed effects are not secondary to an altered state of endoderm. Consistent with our hypothesis that SHF proliferation is a driver of OFT/RV morphogenesis, the length (5/7 embryos) or length plus width (2/7 embryos) of the OFT/RV in *Mesp$^{Cre/+}$/Smad1$^{fl/fl}$* hearts was increased at E9.5 (Figures 6D–6F).

We also assessed whether there was a cell autonomous migration defect in *Nkx2-5*-null SHF cells by examining their ability to be deployed to the OFT/RV in a competitive situation with wild-type cells in embryo chimeras (Figure S4). However, we found no evidence for a defect in this assay.

### Genetic Rescue of OFT Formation in *Nkx2-5*-Null Embryos

To test genetically whether defective OFT development in *Nkx2-5* mutants was due to increased Bmp-Smad signaling, we crossed *Nkx2-5* and *Smad1* mutant mice. Deletion of one or two *Smad1* alleles in *Nkx2-5$^{gfp/gfp}$* embryos produced a progressive increase in SHF proliferation (1.5-fold, p = 0.059 and 3.1-fold, p = 0.004, respectively, compared to *Nkx2-5* nulls; Figure 6C). SHF proliferation in *Nkx2-5/Smad1*-double-null embryos was only 23% less than in wild-type embryos, indicating that most of the antiproliferative effect of increased Bmp2 was mediated by Smad1. We found no precocious differentiation in the SHF in *Nkx2-5* mutants, indicating that the negative effects of increased Bmp2-Smad1 signaling on SHF proliferation were independent of any prodifferentiative activity

of Bmps (Figure S5). *Bmp2* remained upregulated at the pericardial/myocardial boundary in *Nkx2-5$^{gfp/gfp}$/Smad1$^{-/-}$* embryos (Figures 6G–6J), indicating that Nkx2-5-mediated repression of *Bmp2* was independent of Smad1. However, the ectopic expression of *Nkx2-5-GFP* within the head mesenchyme, dorsal foregut, and neural tube of *Nkx2-5* mutants was eliminated by *Smad1* deletion (Figures 6K–6N). Therefore, upregulated Bmp2-Smad1 signaling can account for elevated cardiac specification and reduced SHF proliferation in *Nkx2-5* mutants. We therefore favor the hypothesis that Nkx2-5 acts indirectly to repress progenitor cell specification and proliferation.

In the forming heart, *Wnt11* expression marks the inflow and outflow poles (Figures 6O, O′, and O′′), which are derivatives of the SHF. At E8.5, *Smad1$^{-/-}$* heart tubes were truncated and partially bifid (Figures 6Q and Q′′) (M.J.S., M.B.F., and R.P.H., unpublished data). *Smad1$^{-/-}$* hearts initially expressed *Wnt11* throughout most of the myocardium, suggesting that the primary heart tube in this mutant context is formed predominantly from SHF cells, a possible result of retarded specification of FHF cells and/or increased SHF proliferation. As shown above, *Nkx2-5$^{gfp/gfp}$* hearts have a highly compromised SHF-derived component and accordingly showed downregulated *Wnt11* (Figures 6P, P′, and P′′). *Nkx2-5$^{gfp/gfp}$/Smad1$^{-/-}$* hearts were also partially bifid, but in striking contrast to *Nkx2-5*-null hearts, had greater morphological development of the outflow region, consistent with improved SHF proliferation and deployment, and prominent *Wnt11*-positive outflow and inflow poles (Figures 6R, R′, and R′′).

We next explored whether the persistent expression of cardiac progenitor genes in differentiated myocytes of *Nkx2-5* mutants was also Smad1 dependent. Isl1 was undetectable in the hearts of *Smad1$^{-/-}$* embryos but remained upregulated in the hearts of *Nkx2-5$^{gfp/gfp}$/Smad1$^{-/-}$* embryos (Figures 6S–6V). Thus, Nkx2-5 represses cardiac progenitor genes through multiple targets.

### The Nkx2-5/Bmp2/Smad1 Negative Feedback Loop Is a Molecular Target in CHD

We found that our previously established *Nkx2-5-IRES-cre* knockin allele (Stanley et al., 2002) produced ~50% less Nkx2-5 protein than a wild-type allele at E8.0 (data not shown). *Nkx2-5$^{+/IRES-cre}$* and *Nkx2-5$^{IRES-cre/IRES-cre}$* mice were grossly normal, but embryos carrying one *Nkx2-5-IRES-cre* and one null allele (*Nkx2-5$^{gfp/IRES-cre}$*) were hypomorphic and died postnatally, showing a spectrum of cardiac malformations overlapping the more severe defects seen in humans with *NKX2-5* mutations (Figures 7A–7D). At mid-to-late gestation (E14.5 or E17.5; n = 29), all hypomorphic hearts showed a ventricular septal defect (VSD), malrotation of the OFT vessels, and either DORV or a related condition in which the aorta is positioned above a VSD (overriding aorta OA; Figures 7A–7D and 7Q). Similar malformations were seen at birth. Other frequent abnormalities included interruption of the interatrial septum primum (primum ASD), immature or absent
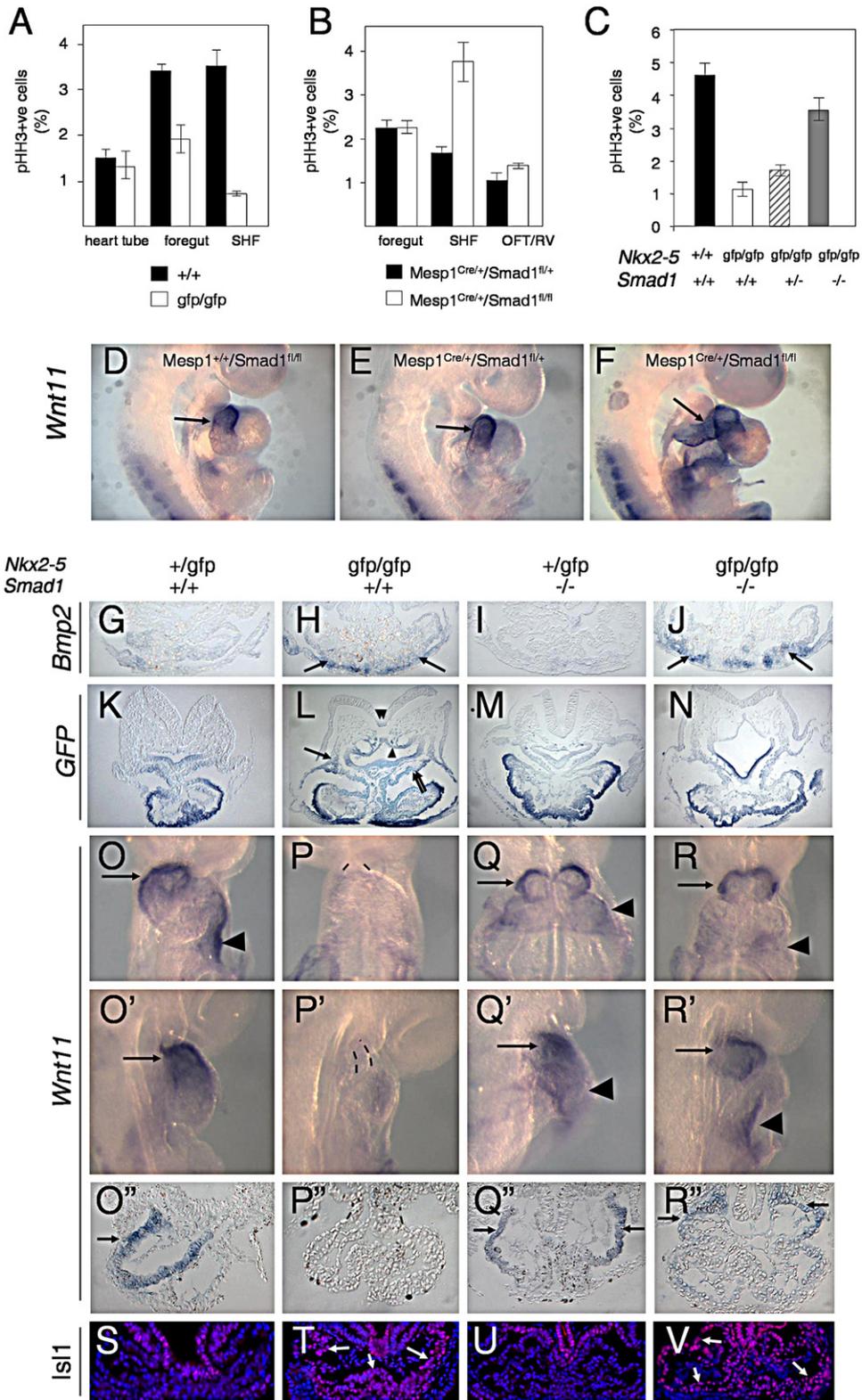
**Figure 6. Nkx2-5 and Smad1 Have Opposing Effects on SHF Proliferation and Deployment**

(A–C) Cell proliferation in the SHF of *Nkx2-5$^{gfp/gfp}$* mutant versus wild-type control (+/+) embryos (A; E8.0, 6–8 ps, C57BL/6 genetic background) and embryos deleted for *Smad1* in mesoderm (*Mesp1$^{Cre/+}$/Smad1$^{fl/fl}$*) versus heterozygous deleted controls (*Mesp1$^{Cre/+}$/Smad1$^{fl/+}$*) (B; E8.0, 6–8 ps, 75%

mitral and tricuspid valves, and thin-walled ventricles (Figures 7D and 7Q–7T and Table S2).

Outflow vessel defects seen in *Nkx2-5* hypomorphs at midgestation were predated by OFT defects in earlier embryos; e.g., the OFT was shorter and thinner and the RV bulge less prominent at E8.5–9.5 compared to *Nkx2-5*[+/IRES-cre] controls (Figures 7E–7H). These OFT defects may be a less severe manifestation of those seen in *Nkx2-5* nulls. Indeed, at late head-fold stages (E7.75), *Bmp2* and Isl1 expressions were elevated in hypomorphs (Figures 7I–7K), as was *Nkx2-5-IRES-cre* expression measured by qRT-PCR (1.4-fold increase, p = 0.041), the latter indicative of cardiac progenitor overspecification. We also examined proliferation in hypomorphs at E8.5 and found a decrease in the SHF of 51% but no effect on OFT/RV cardiomyocytes (Figure 7L; p = 0.024).

Following logic established for null embryos, we asked whether the OFT/RV defects seen in *Nkx2-5* hypomorphs could be rescued by *Smad1* deletion. Deletion of one *Smad1* allele in the germline, or a floxed *Smad1* allele via expression of Cre from the *Nkx2-5-IRES-cre* allele, was sufficient to increase the size of the OFT and RV swelling at E9.5 (Figures 7M and 7N). In conditionally deleted *Nkx2-5*[gfp/IRES-cre]/*Smad1*^{fl/+} embryos at E14.5, malrotation and rightward displacement of the OFT vessels were largely corrected, and in 11 of 13 cases, the aorta was correctly oriented leftwards and connected directly to the LV (p = 0.00024; Table S2 and Figures 7O–7X). VSD and mitral valve dysmorphogenesis was also less severe in *Nkx2-5*[gfp/IRES-cre] hypomorphs with conditionally deleted *Smad1* (p = 0.0022 and p = 0.018, respectively; Table S2 and Figures 7Q–7X). RV size was also improved in some embryos (Figures 7O–7X), and in most, maturation of the tricuspid valve was enhanced and an intact atrial septum was restored, although in isolation these results did not reach significance (p = 0.053 and p = 0.14, respectively; Table S2). Normal OFT vessel connections, intact atrial septa, and improved RV size, AV valve morphology and VSD severity, were also observed in two *Nkx2-5*[gfp/IRES-cre]/*Smad1*^{fl/fl} embryos recovered at E14.5.

## DISCUSSION

In *Nkx2-5* mutants, the upregulation of progenitor signature genes in the SHF and the abnormal persistence of expression in differentiating myocytes indicate a major early role for Nkx2-5 in modulating expression of genes associated with cardiac induction and progenitor cell status. The majority of the progenitor genes affected by loss of Nkx2-5 were expressed in both the FHF and SHF. Expression in the FHF was extremely transient, reflecting the early differentiation of these progenitors in the cardiac crescent. For example, Isl1, previously thought to be SHF specific, was expressed throughout the intraembryonic coelomic lining at E7.5, which contains components of both progenitor fields and is therefore likely to be a pan-cardiac progenitor cell marker in mice. Transient FHF Isl1 expression may account for the dearth of marked FHF descendents in a Cre-fate map (Cai et al., 2003). The expression patterns of progenitor markers define new subpopulations and/or their behavioral states. In the SHF, *Tnc*, *Pdgfra*, and *Igfbp5* were expressed most highly in caudal cells, while anteriorly, expression of *Pdgfra* was restricted to its medial portion (dorsal mesocardium) and that of *Tnc* to the most lateral regions. In contrast to skeletal muscle development, where proliferation ceases as differentiation begins and for which there are relatively clear molecular signatures for progenitors (*Pax3/7*), myoblasts (*MyoD*, *Myf5*) and myocytes (*myogenin* and contractile proteins), cardiac cells continue to proliferate during differentiation. It is possible that some of the identified markers will reflect analogous progressions in the cardiac lineage, although the distinction between progenitor, blast, and differentiated states seem less distinct in cardiac muscle.

### An Nkx2-5/Bmp2/Smad1 Negative Regulatory Circuit Limits Cardiac Specification

We attribute both overspecification and proliferative failure of cardiac progenitor cells in *Nkx2-5* mutants to an overactive Bmp2/Smad1 pathway. Several observations suggested involvement of a long-range diffusible morphogen in the spectrum of defects seen in *Nkx2-5* mutants, including upregulation of *Bmp2* in the cardiac fields and increased levels of phospho-Smad1/5/8 in and beyond the fields. Bmp2-related factors are cardiac-inducing molecules in vertebrates and invertebrates, with *Nkx2-5* and its *Drosophila* homolog *tinman* being direct transcriptional targets of Bmp-Smads (Liberatore et al., 2002; Lien et al., 2002). Therefore, an early and possibly direct function for Nkx2-5 in the cardiac developmental program is to

Quackenbush-Swiss/25% C57BL/6) and for littermate embryos from *Nkx2-5*[+/gfp] X *Smad1*^{+/−} crosses (C, E8.5, 10–12 ps, 50% Quackenbush-Swiss/50% C57BL/6). Shown is the mean percentage of phosphohistone H3[+] (pHH3) cells ± standard deviation (SD), n = 3 for each genotype.

(D–F) ISH for *Wnt11* mRNA highlighting increased OFT/RV (arrows) size in *Mesp1*[Cre/+]/*Smad1*^{fl/fl} embryos versus *Mesp1*^{+/+}/*Smad1*^{fl/fl} and *Mesp1*[Cre/+]/*Smad1*^{fl/+} controls at E9.5.

(G–V) Marker analysis of wild-type, *Nkx2-5*-null, *Smad1*-null, and *Nkx2-5*/*Smad1*-double-null littermate embryos for *Bmp2* (E8.0; G–J), *Nkx2-5-GFP* (E8.25; sections, K–N), *Wnt11* (E8.5; ventral views; O–R; right-hand side views, O′–R′; section through OFT, O″–R″), and Isl1 (E8.25, sections; S–V). Note in Figure 6L the expanded *Nkx2-5-GFP* expression in the dorsal pericardium (double arrows), adjacent mesenchyme (arrow), dorsal foregut (arrowhead), and ventral neural tube (double arrowheads) of *Nkx2-5*-null embryos. In Figures 6O–R″ *Wnt11* expression is indicated in the OFT (arrows) and inflow region (arrowheads) of wild-type (O, O′, and O″), *Smad1*-null (Q,Q′, and Q″), and *Nkx2-5*/*Smad1*-double-null (R, R′, and R″) embryos but is absent from the rudimentary OFT of *Nkx2-5*-null embryos (outlined by dotted lines; P, P′, and P″). Persistent Isl1 expression in cardiomyocytes located in the heart tubes of *Nkx2-5*-null (T) and *Nkx2-5*/*Smad1*-double-null (V) embryos is indicated (arrows).
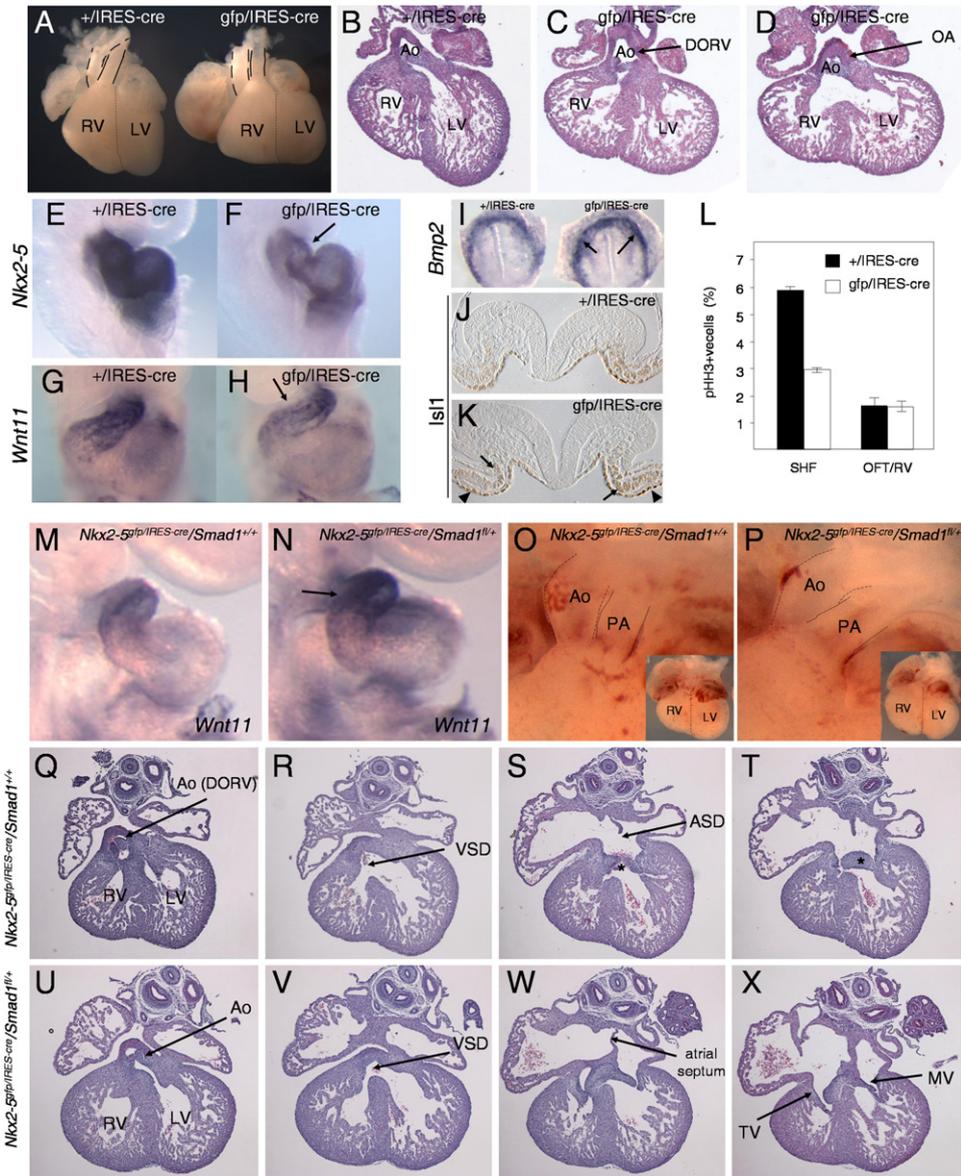
**Figure 7. Genetic Rescue of Congenital Cardiac Malformations in *Nkx2-5* Hypomorphic Embryos**

(A–D) Gross morphology of *Nkx2-5* hypomorphic embryos (gfp/IRES-cre) and littermate controls (+/IRES-cre) at E17.5 showing malrotation and right-ward displacement of the aorta (dashed lines) and pulmonary artery (solid lines) in hypomorphs (A), with sections from E14.5 embryos showing DORV (C) or OA with large VSD (D).

(E–H) Morphological analysis of early OFT development showing compromised outflow development (arrows) in hypomorphs. *Nkx2-5* expression highlights cardiac structures at E8.5 (E and F). *Wnt11* expression marks the RV/OFT at E9.5 (G and H).

(I–K) Molecular analysis of cardiac progenitor cell defects in *Nkx2-5*$^{gfp/IRES-cre}$ hypomorphic embryos and *Nkx2-5*$^{+/IRES-cre}$ controls. Note upregulated expression of cardiac progenitor cell markers *Bmp2* (I; E7.75) and Isl1 (J and K; E7.5) in hypomorphs (arrows, precardiac mesoderm; arrowheads, endoderm).

(L) Decreased proliferation in the SHF of *Nkx2-5*$^{gfp/IRES-cre}$ hypomorphic embryos (E8.5, 10–12 ps, C57BL/6 genetic background, mean ±SD, n = 3 for each genotype).

(M–X) Rescue of early OFT defects in *Nkx2-5*$^{gfp/IRES-cre}$ hypomorphic embryos by deletion of a single allele of *Smad1*. The immature AV cushions in hypomorphs are indicated by an asterisk (S and T). The following abbreviations are used: Ao, aortic root; ASD, atrial septal defect; DORV, double outlet right ventricle; LV, left ventricle; MV, mitral valve; OA, overriding aorta; PA, pulmonary artery; RV, right ventricle; TV, tricuspid valve; VSD, ventricular septal defect.

dampen expression of *Bmp2*, its immediate upstream inducer. Preliminary data show that multiple predicted enhancers of *Bmp2* are normally occupied by Nkx2-5 in cardiac cells, and these warrant further investigation.

The Bmp repressor noggin can stimulate proliferation of SHF cells in vitro (Waldo et al., 2001), and, as shown here genetically, *Smad1* is a critical negative regulator of SHF proliferation in vivo. In contrast to the FHF, SHF cells undergo an extended period of mitotic expansion lasting several days prior to, and overlapping with, their deployment to the OFT/RV and differentiation. The decreased proliferation of the SHF in *Nkx2-5* mutants likely accounts for the progressive normalization in numbers of *Nkx2-5*-GFP$^+$ cells following their initial overspecification. A model for the feedback loop involving Nkx2-5, Bmp2, and Smad1 and its role in cardiac specification, progenitor gene expression, and proliferation is presented in Figure S6. In the context of developmental time, the repressive action of Nkx2-5 on Bmp2/Smad1 orchestrates the transition between a period of cardiac induction and one of progenitor proliferation, a negative feedback that is likely to be a fundamental property of cardiac developmental circuitry in all vertebrates. Given that Nkx2-5 levels increase from the progenitor to the differentiated state, the negative feedback pathway may be an example of how the number of progenitor cells in a field is controlled by the degree of differentiation—a paradigm that could apply to adult stem cells and organ regeneration. In this paradigm, Nkx2-5 acts as both the sensor of differentiation and as the arbiter of repression. Because the negative feedback loop balances the number of cardiac progenitors through opposing effects on induction and proliferation, it is self regulating and thereby shows features of robustness, an essential component of developmental systems.

Coexpression of genes defining cardiac progenitor cells, including *Isl1*, with cardiomyogenic genes in *Nkx2-5*-null heart tubes demonstrates that Nkx2-5 also plays a critical role as a molecular switch in ensuring spatial and temporal discrimination of the progenitor and differentiating states. However, persistent expression of Isl1 (and presumably other progenitor genes) in the forming heart tube of *Nkx2-5* mutants was not rescued by *Smad1* deletion, suggesting that Nkx2-5-dependent negative feedback targets multiple sets of genes with distinct functional consequences for heart development.

### A Cardiac Progenitor Cell Defect May Underlie *NKX2-5*-Related CHD

The decrease in SHF proliferation in *Nkx2-5*-null embryos has potential ramifications for our understanding of human *NKX2-5*-related CHD. We describe a hypomorphic *Nkx2-5* model in which animals survived until birth with a spectrum of cardiac anomalies overlapping the more severe malformations seen in some humans with *NKX2-5* mutations. Of note is the high penetrance of OFT defects accompanied by large VSD in this model. These are not seen in heterozygous *Nkx2-5*-null mice (Biben et al.,

2000) and are sporadic findings in human patients (McElhinney et al., 2003), yet are fully penetrant in a heterozygous dominant-negative model of Nkx2-5 deficiency (Elliott et al., 2006). These findings highlight the fine balance between the effective activity of Nkx2-5 and CHD outcome. In humans, the subtle effects of modifier genes on NKX2-5 levels may explain, in some cases, the variable phenotypes seen in patients with *NKX2-5* mutations.

Our data provide support for correlative evidence that SHF proliferation is a critical determinant of SHF deployment and the size and morphology of the OFT/RV. In chick embryos, physical ablation of either the SHF or neural crest, which supports SHF development, leads to a severely decreased contribution of cells to the OFT (Ward et al., 2005; Yelbuz et al., 2002). This produces abnormal looping and rightward displacement of the OFT, manifesting later as an OA (Ward et al., 2005; Yelbuz et al., 2002). *Nkx2-5* hypomorphs showed diminished SHF proliferation, and OFT size and morphology were compromised as early as E8.5, leading to abnormal displacement of outflow vessels to the right, OA, and, in more severe cases, DORV. These OFT/RV malformations were partially rescued by deletion of one or both *Smad1* alleles. However, not all cardiac defects were rescued in *Nkx2-5* hypomorphs by *Smad1* deletion. In particular, small VSDs and ventricular chamber defects persisted. These are likely caused by a reduction in chamber differentiation, in which Nkx2-5 is known to have a direct role (Pashmforoush et al., 2004).

OFT defects are among the most severe cardiac congenital malformations and often require surgical intervention in the first year of life. Our study suggests that SHF genes and those involved in the Nkx2-5/Bmp2/Smad1 negative feedback loop will be molecular targets and modifiers in CHD. The discovery of this pathway that balances cardiac progenitor specification and proliferation now provides a basis for further mechanistic investigations in this pathological context, as well as in that of the deployment of stem cells that underlies the formation of the heart and, eventually, also its repair.

### EXPERIMENTAL PROCEDURES

#### Microarray
Amplified RNA from FACS-sorted Nkx2-5$^{gfp/+}$ and Nkx2-5$^{gfp/gfp}$ cells was labeled with Cy3 or Cy5 and hybridized to cDNA arrays. For the full details (MIAME-compliant) of the clone sets used, array printing, sample preparation, labelling, and hybridization, see ArrayExpress database, accession numbers E-MEXP-533 and -534.

#### Gene/Protein Expression
Controls used in all experiments were somite-matched littermates. ISH was as described (Biben and Harvey, 1997). Probes are described in Table S1. For whole-mount immunohistochemistry, PFA-fixed embryos were treated with acetone for 7 min at −20°C, then blocked for 1 hr in 3% skim milk. For Isl1 and pSmad1, embryos were heated at 95°C for 8 min in unmasking solution (Vector Laboratories) prior to acetone fixation. Antibodies used were against Pdgfra (APA5, PharMingen), TER-119 (PharMingen), Isl1 (supernatant 39.4D5, raised against chick Isl1 C terminus, developed by Thomas Jessell, and

obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa; and goat anti-Isl1, raised against full-length human Isl1, GT15051, Neuromics), phospho-Smad1/5/8 (9511S, Cell Signaling), β-galactosidase (gift from JF Nicolas, Pasteur Institute), sarcomeric myosin heavy chain (MF20, Developmental Studies Hybridoma Bank), and phosphohistone H3 (06-570, Upstate). Quantitative RT-PCR was as described (Stennard et al., 2005) (primers available on request). For statistical methods, see figure legends.

## Supplemental Data

Supplemental Data include six figures, two tables, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/128/5/947/DC1/.

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**Accession Numbers**

The ArrayExpress accession numbers for the microarray data reported in this paper (including the MIAME-compliant details of array construction and microarray experimental procedures) are E-MEXP-533 and E-MEXP-534.