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Pitx2 regulates cardiac left–right asymmetry by patterning second cardiac lineage-derived myocardium

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Abstract

Current models of left–right asymmetry hold that an early asymmetric signal is generated at the node and transduced to lateral plate mesoderm in a linear signal transduction cascade through the function of the *Nodal* signaling molecule. The *Pitx2* homeobox gene functions at the final stages of this cascade to direct asymmetric morphogenesis of selected organs including the heart. We previously showed that *Pitx2* regulated an asymmetric pathway that was independent of cardiac looping suggesting a second asymmetric cardiac pathway. It has been proposed that in the cardiac outflow tract *Pitx2* functions in both cardiac neural crest, as a target of canonical Wnt-signaling, and in the mesoderm-derived cardiac second lineage. We used fate mapping, conditional loss of function, and chimera analysis in mice to investigate the role of *Pitx2* in outflow tract morphogenesis. Our findings reveal that *Pitx2* is dispensable in the cardiac neural crest but functions in second lineage myocardium revealing that this cardiac progenitor field is patterned asymmetrically.

Keywords

Left-right asymmetry; Secondary heart field; Homeobox

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Introduction

Left–right asymmetric morphogenesis (LRA) has long been recognized to play a critical role in cardiac morphogenesis (Brown and Anderson, 1999). However, the mechanisms that integrate LRA into overall cardiac morphogenesis are poorly understood. The *Pitx2* homeobox gene has an essential role in the late aspects of LRA (reviewed in (Franco and Campione, 2003)).

Inactivation of *Pitx2* in mice revealed an important role for *Pitx2* in many aspects of cardiac development. For example, *Pitx2* null mice had severe defects in valve formation, sinuatrial morphogenesis, and alignment of the arterio-ventricular connections such as doublet outlet right ventricle (DORV) and transposition of the great arteries (TGA) (Kitamura et al., 1999; Liu et al., 2001). Growth of the RV myocardium was also diminished resulting in RV hypoplasia (Kioussi et al., 2002; Kitamura et al., 1999; Liu et al., 2001, 2002). Surprisingly, *Pitx2* null mice had correct, dextral looping of the heart, a cardinal feature of asymmetric morphogenesis. Thus, the *Pitx2* null cardiac phenotype, while uncovering a critical role for *Pitx2* in asymmetric cardiac development, raised a number of important questions regarding the relationship of early asymmetric signaling pathways to organ-specific asymmetry (Harvey, 2002).

Previous models based on the phenotypes of *iv* mutant mice and human patients with heterotaxia, or discordant asymmetric organ morphogenesis, provided a framework for understanding the interaction of early, pre-organogenesis embryonic asymmetry with each organ primordium (Brown and Wolpert, 1990). In the biasing model of asymmetry, an early signal in the embryo induces an asymmetric signaling cascade that serves to bias or globally coordinate organ asymmetry. The local generation of asymmetry in the individual organ is an all or none random event. However, the biasing model of asymmetry failed to explain the phenotype of *Pitx2* null mice that had laterality defects in a subset of cardiac cells.

Recent work, using retrospective clonal analysis in mouse embryos, defined two cardiac progenitor lineages. The first lineage, corresponding to cells within the primary heart field (PHF), uniquely contributes to the left ventricular (LV) myocardium while the second lineage contributes to outflow tract (OFT) and right ventricular (RV) myocardium. Both the first and second lineages contribute cells to the atria and AV canal myocardium (Meilhac et al., 2004a,b). Further functional evidence for the existence of the second lineage was provided by the analysis of *Isl1* mutant embryos that had severe defects in the OFT and inflow regions of the heart. *Isl1* is expressed in cardiac progenitors but is excluded from the majority of cardiac cells with the exception of a subpopulation of adult cardiac progenitor cells (Cai et al., 2003; Laugwitz et al., 2005).

A subpopulation of the second lineage, the secondary (or anterior) heart field (SHF), is derived from the splanchnic and branchial arch mesoderm and contributes to OFT myocardium (Cai et al., 2003; Kelly and Buckingham, 2002; Mjaatvedt et al., 2001; Waldo et al., 2001). The SHF was originally defined in chick embryos using fate mapping and lineage tracing approaches (Mjaatvedt et al., 2001; Waldo et al., 2001). An enhancer trap into the *Fgf10* locus provided evidence for the existence of the SHF in mice (Kelly et al.,

2001). These findings raised the hypothesis that asymmetric cardiac patterning information may be imported into the heart through the movement of cells into distinct regions of the developing heart (Kioussi et al., 2002; Liu et al., 2002).

Pitx2c, the LRA-specific isoform, is expressed in left lateral plate mesoderm, left side of the branchial arch and splanchnic mesoderm, OFT myocardium, and the left atrium (Kitamura et al., 1999; Liu et al., 2002; Schweickert et al., 2000). The *Pitx2c* expression pattern is consistent with the hypothesis that *Pitx2c* may pattern the second lineage. Expression of the other two *Pitx2* isoforms, *Pitx2a* and *Pitx2b*, has not been detected in the left lateral plate mesoderm or heart. Recent work provided evidence that the *Pitx2a* isoform is expressed in the CNC and is a target of a *Wnt-β catenin* signaling pathway in the OFT (Kioussi et al., 2002). A strong genetic interaction with *Disheveled 2 (Dvl2)*, also proposed to have a function in CNC, was uncovered (Hamblet et al., 2002; Kioussi et al., 2002). Taken together, these findings suggest that the *Pitx2*-regulated genetic program in the SHF and CNC regulates asymmetric cardiac morphogenesis.

In this work, we focused on the role of *Pitx2* in OFT morphogenesis. We directly investigated the function of *Pitx2* in the SHF and CNC using conditional gene targeting in mouse embryos to delete all *Pitx2* isoforms in the second cardiac lineage and CNC. Our data, indicating that *Pitx2* functions in the SHF and is dispensable in the CNC, provides definitive evidence that the SHF is an important source of cardiac left– right asymmetry. Our findings also uncover sequential functions for *Pitx2* in expansion and then remodeling of RV and OFT myocardium.

Materials and methods

Whole mount in situ hybridization and morphometric analysis

Embryos were fixed in 10% formalin and kept in 100% methanol until used. Whole mount in situ hybridization was performed as previously described (Lu et al., 1999). For all experiments, at least three embryos were used for each probe at each time point examined. Probes for in situ: *CrabpI*, *Ap2*, and *Isl1* probes have been described (Cai et al., 2003; Maden et al., 1992; Mitchell et al., 1991). For morphometric analysis, mutant and control embryos were photographed together and analyzed using NIH image freeware (version 1.63). Data were compiled into an Excel file and analyzed statistically.

LacZ staining and histology

For histology, embryos were fixed overnight in Bouin's fixative or buffered formalin, dehydrated through graded ethanol and embedded in paraffin. Sections were cut at 7–10 μm and stained with H&E. Staining for β-galactosidase (LacZ) was as previously described (Lu et al., 1999).

Proliferation assay

9.5 dpc embryos were formalin-fixed, paraffin-embedded and cut at 5 μm. Proliferating cell nuclear antigen (PCNA) staining was performed according to the manufacturer's instructions (Zymed).

Mouse alleles and nomenclature

To generate the *Pitx2*^{flox} allele, a *Pitx2* hypomorphic 3 LoxP allele previously generated was crossed to a CMV Cre transgenic line that is a germline mosaic deletor (Gage et al., 1999). Mice that were mosaic for the 3LoxP and flox alleles were obtained and backcrossed to C57Bl/6J wild type mice to generate mice exclusively carrying the *Pitx2*^{flox} allele in the germline. The *Pitx2*^{null}, previously called *Pitx2* δ*abc*^{null}, and *Pitx2*^{cre}, previously called *Pitx2* δ*abc*^{cre}, alleles have been previously described (Liu et al., 2003; Lu et al., 1999). The Pitx2ab isoform specific deletion, previously called *Pitx2 δab*, has been described (Liu et al., 2001).

Generation of the Pitx2^{creERT2} allele

We constructed a targeting vector that introduces the *cre recombinaseERT2 neofrt* fusion cassette into the *Pvu*II and *Nru*I sites in the *Pitx2* fifth exon, a strategy that is similar to that used to generate the δ*abc*^{creneo} allele (Liu et al., 2003). The *Pitx2*^{creERT2neo} targeting vector, that contains approximately 7 kb of *Pitx2* homologous sequences included the *PGKneomycin* resistance cassette flanked by Frt sites for removal of the *PGKneomycin* by flp recombinase. The targeting vector was electroporated into the AK7 ES cell line, recombinant ES clones were identified by Southern blot and injected into 3.5 dpc wild type C57bl/6J mouse embryos to generate chimeric and F1 heterozygous mice carrying the *Pitx2*^{creERT2} allele. The *Pitx2*^{creERT2} allele was maintained on a mixed 129/Sv × C56BL/6J genetic background.

Generation of aggregation chimeras

ICR (CD-1; Harlan Sprague–Dawley, Indianapolis, IN) and *Pitx2* mutant 2.5 dpc embryos were harvested in M2, supplemented with 20 mM HEPES (Specialty Media, Phillipsburg, NJ). Morula stage embryos were flushed from oviducts, rinsed thoroughly in M2, and placed in 20 μl KSOM drops overlaid with oil. Morulae were placed into Acidic Tyrode's and gently pipetted up and down until the zona pellucida dissolved. Morulae were rinsed and transferred to the holding drop in the aggregation dish and placed in an incubator at 37°C, 5% CO_2 for overnight culture and then transferred to pseudopregnant females.

Casting dye injection

Embryos were harvested and yellow casting dye (Connecticut Valley Biological Supply Co.) was injected into the ventricle using pulled glass and fixed in buffered formalin, dehydrated, and cleared in benzyl alcohol; benzyl benzoate (BABB).

Results

Cardiac neural crest markers are unaltered in Pitx2^{null} mutants

To investigate the hypothesis that *Pitx2* played an important role in CNC development, we analyzed CNC markers in *Pitx2*^{null} mutant embryos. Expression of the CNC markers *Crabp1* and *Ap2* was detected at normal levels in the CNC of *Pitx2*^{null} mutant embryos suggesting that cardiac neural crest development was intact in *Pitx2*^{null} mutant embryos at 9.5 dpc (Figs. 1A–D).

We performed fate mapping with the *Wnt1 cre* transgenic line and the *Rosa26 reporter* (*R26R*) allele (Jiang et al., 2000; Soriano, 1999). Although we had previously shown that *Wnt1cre* marked daughter cells contribute normally to the OFT of *Pitx2c*−/− embryos, the question has been raised that this may be due to functional compensation by the *Pitx2a* or *Pitx2b* isoforms (Franco and Campione, 2003). Examination of CNC contribution to the OFT of *Pitx2null* mutant embryos revealed that the CNC migrated normally into the OFT in the absence of *Pitx2* (Figs. 1E–J). Taken together, these data suggest that the migration and specification of CNC is intact in *Pitx2null* mutant embryos.

We also evaluated the status of canonical Wnt-signaling in the OFT cushions of *Pitx2null* embryos. Previous experiments have shown that inactivation of *β catenin* in the CNC resulted in severe defects in OFT development (Kioussi et al., 2002). The *TopGal* reporter mouse strain harbors a transgene containing multimerized Lef/TCF binding sites upstream of a *LacZ* reporter and has been shown to be an accurate measure of Wnt signaling (Das Gupta and Fuchs, 1999). In wild type embryos, the *TopGal* reporter directed high levels of LacZ activity in the OFTcushions of both wild type and *Pitx2null* embryos (Figs. 1K, L and (Gitler et al., 2003)). This indicates that canonical Wnt-signaling in the OFT cushions is intact in the *Pitx2null* mutant embryos.

Tissue-specific Pitx2 inactivation using the Wnt1cre transgene reveals that Pitx2 is dispensable for CNC development

We next performed a direct, functional investigation of *Pitx2* in the CNC. To do this, we generated a conditional null allele of *Pitx2*, the *Pitx2flox* allele (Gage et al., 1999 and Materials and methods). To ablate all isoforms of *Pitx2* specifically in the CNC, we used the *Wnt1cre* transgenic line, a well characterized neural crest Cre recombinase driver, that has been successfully used to ablate other genes, such as β catenin, in neural crest (Brault et al., 2001; Jiang et al., 2000). We intercrossed the *Pitx2flox* allele to *Wnt1cre; Pitx2null* compound heterozygous mice, a strategy requiring recombination at a single *Pitx2* allele to generate a *Pitx2* null cell. Examination of multiple litters revealed that *Wnt1 cre*; *Pitx2null/flox (n/f)* mutants were viable, fertile, and lived for at least 1 year without apparent difficulty.

To investigate the hypothesis that the *Wnt1cre; Pitx2 (n/f)* mutant embryos had subtle cardiac defects that did not influence viability, wild type and *Pitx2* mutant embryos were examined histologically in a blinded fashion. Serial histologic sections indicated that *Wnt1cre; Pitx2 (n/f)* mutant embryos ($n = 5$) had normal cardiac structures when analyzed at 16.5 dpc. In *Wnt1cre; Pitx2 (n/f)* embryos, we observed correct alignment of the aorta (Figs. 2A, C, E, G) and pulmonary trunk (Figs. 2B, D, F, H). Morphology of the conotruncal valves was normal in *Wnt1cre; Pitx2 (n/f)* embryos (compare Figs. 2A, B with C, D). Furthermore, ventricular septation was also normal in the *Wnt1cre; Pitx2 (n/f)*embryos (Figs. 2A, C, E, G).

One possible explanation for absence of a phenotype in the *Wnt1cre; Pitx2 (n/f)* embryos was that the *Wnt1cre* transgenic line failed to completely delete *Pitx2* in the CNC. The current model suggests that *Pitx2a* is the CNC-specific *Pitx2* isoform (Kioussi et al., 2002). We evaluated OFT development in *Pitx2a, b*−/− mutant embryos that we had previously generated (Liu et al., 2001). Consistent with the phenotype of *Wnt1cre; Pitx2 (n/f)* embryos,

the *Pitx2a, b*−/− mutant embryos also had a normal OFT (Fig. S1). Taken together, these data indicate that *Pitx2* is not required in the CNC for normal OFT development.

Lineage tracing and fate mapping revealed that Pitx2 daughter cells populate the conotruncal myocardium

Because the OFT derives from a complex mixture of cells with distinct lineages including the CNC and SHF component of the second lineage, unequivocal identification of the *Pitx2*-expressing cell lineage is inherently difficult using standard expression analysis. To provide supportive evidence that *Pitx2* expressing cells in the OFT were of SHF origin, we employed a genetic cell labeling method based on Cre-Lox technology (Nagy, 2000).

We first used the $Pitx2^{Cre}$ knock in allele to perform a lineage tracing experiment to follow *Pitx2* daughter cells in the OFT (Liu et al., 2003). The $Pitx2^{Cre}$ allele is a null allele of *Pitx2* that directs Cre activity in cells that are fated to express all isoforms of *Pitx2*, including the *Pitx2c* cardiac isoform (Fig. 3A). The $Pitx2^{Cre}$;*R26R* compound heterozygous embryos were analyzed at 11.5 dpc and 14.5 dpc (Figs. 3B, C). There was a slight delay in Cre activity in the 11.5 dpc OFT most likely as a result of the extra time required to accumulate enough Cre protein to induce recombination in the cell (Nagy, 2000). Nonetheless, LacZ-marked *Pitx2* daughter cells were easily detected in the OFT, as well as in the RV consistent with the hypothesis that *Pitx2* functioned in the second lineage (Meilhac et al., 2004a; Zaffran et al., 2004).

Sections through the 11.5 dpc $Pitx2^{Cre}$; *R26R* embryos revealed that *Pitx2* daughter cells were confined to the second lineage-derived OFT myocardium and endocardium (Fig. 3D). Previous work has shown that the OFT myocardium and a subpopulation of the endocardium are derived from *Isl1*-expressing and *Mef2c*-expressing cells within the second lineage (Cai et al., 2003; Verzi et al., 2005). It notable that in both the 11.5 dpc and 14.5 dpc embryos a small number of *Pitx2* daughters contributed to the proximal left ventricle. This may indicate that a subset of first lineage cells express *Pitx2.* Alternatively, it is conceivable that some *Pitx2* daughters move from the RV to the proximal LV. Further work will be required to investigate these possibilities.

To perform more precise fate mapping, we generated a $Pitx2^{CreERT2}$ knock-in allele that was also a null allele of *Pitx2* (Fig. 3E). In the $Pitx2^{CreERT2}$ allele, we introduced an IRES Cre-mutant estrogen receptor ligand binding domain (ER^{T2}) fusion cassette into *Pitx2* exon 5. The $CreERT^2$ fusion protein is expressed in cells fated to express *Pitx2* but is maintained in an inactive state in the cytoplasm. Upon injection of 4-OH tamoxifen (4-OHT) that binds to $CreERT^2$, the $CreER^{T2}$ fusion peptide translocates to the nucleus and activates the *R26R* reporter allele. The $CreER^{T2}$ version binds 4-OHT with high affinity allowing for labeling of cells over a short time interval for fate mapping studies of *Pitx2* daughter cells (Indra et al., 1999). After 4-OHT is metabolized and cleared from the circulation, $CreER^{T2}$ reverts to an inactivate state (Hayashi and McMahon, 2002).

We labeled cells with the $Pitx2^{CreERT2}$ allele at 9.5 and 10.5 dpc. Pregnant females from intercrosses between $Pitx2^{CreERT2}$+/− and R26R+/− mice were injected with 4-OHT at 9.5 and 10.5 dpc. Because *Pitx2* has been reported to be expressed in the CNC cells of the

branchial arch mesenchyme at these stages, we predicted that there would be a large contribution of *Pitx2* daughters to the OFT cushion mesenchyme (Hamblet et al., 2002; Kioussi et al., 2002). Injected embryos were harvested at 12.5 dpc and stained for LacZ activity. We found that *Pitx2* daughter cells contributed almost exclusively to the OFT and RV myocardium (Figs. 3F–I).

We noted that lineage tracing with the $Pitx2^{cre}$ allele revealed that a few *Pitx2* daughter cells contributed to OFT cushion mesenchyme. The OFT cushion mesenchyme receives a contribution from both the CNC and OFT endocardium. Endocardial cells of the OFT are derived from the SHF and undergo an epithelial–mesenchymal transition to form cushion mesenchyme (Cai et al., 2003; Eisenberg and Markwald, 1995). Our observation that *Pitx2* daughters differentiate into OFT endocardium is consistent with *Pitx2* daughters contributing to cushion mesenchyme (Fig. 3D). Taken together with the $Wnt1^{cre}$ inactivation data, these findings indicate that *Pitx2* daughter cells primarily contribute to the OFT and RV myocardium suggesting that *Pitx2*-expressing cells are located in the SHF rather than in the CNC.

Inactivation of Pitx2 in the SHF results in severe cardiac defects

To investigate directly *Pitx2* function in the SHF-component of the second lineage, we used the $Pitx2^{flox}$ allele and the $Mef2c^{cre}$ and $Isl1^{cre}$ drivers to inactivate *Pitx2* in the SHF. The $Mef2c^{cre}$ transgenic line directs Cre activity to the anterior aspect of the second lineage while the Isl^{cre} allele directs Cre activity in a broader domain of the second lineage (Cai et al., 2003; Verzi et al., 2005). We used two cre drivers for this experiment in order to make firmer conclusion about *Pitx2* in the SHF. In addition, the $Isl1^{cre}$ allele is an *Isl1* null allele that may complicate data interpretation due to the potential for a genetic interaction between *Isl1* and *Pitx2*. The $Mef2c^{cre}$ driver, a randomly integrated transgene that is viable when homozygous, does not have the potential for a genetic interaction with *Pitx2*. Intercrosses between $Isl1^{cre}$; $Pitx2^{null}$ or $Mef2c^{cre}$; $Pitx2^{null}$ and $Pitx2^{flox}$ females resulted in embryos that were deficient for *Pitx2* in the SHF. We focused our analysis on the OFT and performed casting dye injections in wild type, *Pitx2* SHF deficient, and $Pitx2^{null}$ embryos. At 13.5 dpc, we found that OFT alignment in both the $Isl1^{cre}$; *Pitx2 n/f* and the $Mef2c^{cre}$*; Pitx2 n/f* mutant embryos was severely defective. All embryos that were deficient for *Pitx2* in the SHF had DORV or TGA and were phenotypically similar to the OFT of $Pitx2^{null}$ embryos (Figs. 4A–H).

We performed histological analysis at 13.5 dpc and 14.5 dpc on wild type and $Mef2c^{cre}$*;Pitx2 n/f* mutant embryos ($n = 6$, Fig. 5). Parasagittal sections through 13.5 dpc embryos revealed severe defects in alignment of the OFT in $Mef2c^{cre}$*;Pitx2 n/f* mutant embryos (Figs. 5A–C). At 14.5 dpc, parasagittal and transverse sections revealed that the pulmonary trunk and aorta were in the same plane of section and drained the right ventricle consistent with DORV (Figs. 5D–I). Taken together, these data reveal that *Pitx2* deficiency in the SHF results in OFT defects that are a phenocopy of the $Pitx2^{null}$ phenotype (Kioussi et al., 2002; Liu et al., 2001).

SHF is specified correctly in Pitx2 mutants but outflow tract expansion is defective

The OFT defects observed in the *Pitx2* mutant embryos could be due to defects in undifferentiated SHF cells or in SHF-derived myocardium of the OFT and RV. We performed a fate mapping experiment with the $Isl1^{cre}$ allele to follow the fate of SHF cells in $Pitx2^{null}$ mutant embryos and examined expression of *Is1* in the SHF of $Pitx2^{null}$ mutants (Figs. 6A–D). At 9.5 dpc, fate mapping showed that contribution of *Isl1* daughter cells to the *Pitx2* mutant distal OFT was similar to that of the control embryo, however, in the proximal OFT LacZ-staining was reduced (Figs. 6A, B). This suggested that there were fewer *Isl1* daughter cells in the OFT myocardium of $Pitx2^{null}$ mutant embryos. To establish this more firmly, we performed morphometric analysis on the OFT of $Pitx2^{null}$ and control embryos. Morphometric analysis of eleven $Pitx2^{null}$ mutant embryos indicated that the OFT and prospective RV was smaller than eleven control 9.5 dpc embryos (Fig. 6E).

To address the hypothesis that the OFT deficiency could be secondary to a defect in the SHF, we examined expression of *Isl1* in $Pitx2^{null}$ mutant embryos (Figs. 6C, D). In the $Pitx2^{null}$ mutant, *Isl1* expression was unchanged when compared to control embryos (Figs. 6C, D). Similar expression of *Isl1* in the $Pitx2^{null}$ and control embryos suggested a proliferation defect in OFT myocardium of $Pitx2^{null}$ embryos. We examined cell proliferation in the OFT myocardium of $Pitx2^{null}$ mutant embryos at 9.5 dpc. Consistent with a requirement for *Pitx2* in OFT myocardial expansion, we detected significantly reduced PCNA positive cells in the $Pitx2^{null}$ OFT myocardium (Figs. 6F–H). Taken together, these data indicate that *Pitx2* is required for proliferation of a subpopulation of the proximal OFT myocardium.

Deployment of Pitx2 daughter cells in the proximal OFT is defective

Previous work suggested that *Pitx2* may also function at stages of OFT remodeling (Liu et al., 2002). Lineage tracing with the germline $Pitx2^{cre}$ allele in control embryos indicated that at 12.5 dpc, *Pitx2* daughters normally contributed to the proximal OFT and RV myocardium. In the proximal OFT, *Pitx2* daughters were found within the proximal myocardium overlying both the PT and aorta (Fig. 7A). In contrast, *Pitx2* mutant daughter cells were observed in the proximal OFT, however, contribution to the proximal myocardium of the aorta was limited (Fig. 7B). At 14.5 dpc and 15.5 dpc, a similar defect in *Pitx2* daughter contribution was observed in mutant embryos (Figs. 7C–F). These findings suggest that *Pitx2* functions in local expansion or rotation of proximal OFT myocardium.

Pitx2 chimera analysis uncovers a late function for Pitx2 in OFT remodeling

To investigate *Pitx2* function at later stages of OFT morphogenesis, we used chimera analysis. Although we had uncovered a function for *Pitx2* in expansion of OFT myocardium, we reasoned that analysis of *Pitx2* daughters in the context of a chimera would provide insight into the role of *Pitx2* in OFT remodeling. Our method for generating chimeras was a modification of previous methods (Tam and Rossant, 2003). Using the $Pitx2^{cre}$ and *R26R* alleles to mark *Pitx2* daughter cells, we generated $Pitx2^{cre}$;$Pitx2^{null}$; *R26R* and control $Pitx2^{cre}$+/−; *R26R* aggregation chimeras. Chimeric embryos were recovered at 13.5 dpc and pcr genotyped to detect the presence of the $Pitx2^{cre}$ and $Pitx2^{null}$ alleles. The $Pitx2^{cre}$ and *R26R* alleles resulted in exclusive marking of *Pitx2* daughter cells and allowed direct visualization of *Pitx2* daughters in the presence and absence of *Pitx2* function.

We analyzed eleven control chimeras at 13.5 dpc by whole mount LacZ staining. *Pitx2* daughters contributed extensively to the proximal OFT myocardium, the RV myocardium and the sulcus separating the aorta and pulmonary trunk in the distal and proximal OFT (Figs. 7G–I). Consistent with the lineage tracing in the germline embryos, *Pitx2* daughters contributed to myocardium at the base of the pulmonary trunk and aorta in chimeras.

We examined chimeras with different percentage contribution of $Pitx2^{cre}$;$Pitx2^{null}$ mutant cells. In higher percentage $Pitx2^{cre}$;$Pitx2^{null}$ chimeras ($n = 6$), OFT morphogenesis was disrupted with abnormal ventriculo-arterial alignment, approximating the phenotype observed in the $Pitx2^{null}$ mutant embryos (Figs. 7J, K). Notably in these high percentage chimeras, *Pitx2* mutant daughters were evenly distributed over the proximal myocardium of the aorta and pulmonary trunk (Figs. 7J, K). Thus, in contrast to the germline mutant embryos, *Pitx2* mutant daughters were competent to contribute to the proximal myocardium of the aorta. This suggests that defective contribution to proximal OFT myocardium observed in the $Pitx2^{null}$ germline mutants may be secondary to the earlier myocardial growth defect observed at 9.5 dpc. Alternatively, *Pitx2* may regulate the expression of a signaling molecule in proximal myocardium.

In all chimeras derived from $Pitx2^{cre}$;$Pitx2^{null}$ cells, a large concentration of *Pitx2* mutant daughter cells abnormally persisted in the ventral, proximal myocardium between the great vessels at the ventriculo-arterial junction (Figs. 7J, K, M). We examined chimeras that had a low contribution of *Pitx2* mutant daughter cells (Figs. 7L, M). In these lower percentage chimeras, that had normal OFT anatomy, the accumulation of *Pitx2* mutant daughters was still observed in the proximal OFT. Sections through similar percentage chimeras also showed persistence of mutant cells compared to controls (Figs. 7N, O). These findings indicate that *Pitx2* has an autonomous function in remodeling of the OFT myocardium.

Discussion

The findings presented here indicate that *Pitx2* function in the SHF-derived OFT myocardium is required for correct arterio-ventricular alignment. Our conditional gene inactivation and fate mapping studies show that *Pitx2* functions in the OFT myocardium rather than the CNC. The marker analysis and cell proliferation studies reveal that in $Pitx2^{null}$ embryos the OFT myocardium has a proliferation defect. Moreover, analysis of *Pitx2* mutant cells in chimeric embryos suggests that *Pitx2* has an autonomous function in OFT remodeling. Our findings show that *Pitx2* functions in the OFT myocardium to regulate later stages of OFT growth and morphogenesis and directly link the *Pitx2*-dependent LRA pathway to second lineage myocardium.

Pitx2 in the CNC and SHF

A strong genetic interaction between *Pitx2* and *Disheveled 2* (*Dsh2*) was previously interpreted as evidence that *Pitx2* was functioning in the CNC (Hamblet et al., 2002; Kioussi et al., 2002). *Pitx2* expression was shown in *Wnt1 cre; R26R*-labeled cells in the fourth and sixth branchial arches, and explants showed co-expression of *Pitx2* and a CNC marker (*connexin-43*) in 29% of cells examined (Kioussi et al., 2002). In addition, tissue-specific inactivation of β *catenin* using the $Wnt1^{cre}$ transgene provided supportive evidence that a

canonical Wnt-signaling pathway was involved in CNC development. However, recent work has uncovered the existence of the SHF that also contributes extensively to the OFT (Buckingham et al., 2005).

We provide three lines of evidence that support our proposal that *Pitx2* functions in the SHF rather that the CNC. First, the conditional gene targeting data indicate that removal of *Pitx2* from CNC results in a normal OFT while deletion from the SHF phenocopies the $Pitx2^{null}$ mutant phenotype. While we cannot unequivocally rule out the possibility that ablation of *Pitx2* with the $Wnt1^{cre}$ transgene is incomplete, our second line of evidence argues against this possibility. Our data indicate that germline inactivation of *Pitx2a*, the proposed CNC-specific *Pitx2* isoform, also results in a normal OFT. Lastly, our fate mapping and lineage tracing experiments indicate that the *Pitx2* lineage contributes primarily to cardiac derivatives that are known to be derived from the SHF. Based on these data, we propose that *Pitx2* function resides within SHF-derived cells (Buckingham et al., 2005).

Recent findings from other labs also support our proposal. For example, recent work has shown that *Dsh2* is expressed not in CNC-derived structures but in OFT myocardium — a SHF derivative (Phillips et al., 2005). If the genetic interaction between *Pitx2* and *Dsh2* is due to a shared cell-autonomous function then this would indicate that *Pitx2* functions in the OFT myocardium.

Our findings, together with previous work, implicate Wnt-signaling in the SHF in addition to its known importance in the CNC (Kioussi et al., 2002). *Pitx2* expression in the OFT is severely reduced in the *Dsh2* mutant embryos (Hamblet et al., 2002). Furthermore, *Pitx2* OFT expression is reduced in mice that are deficient for *Smarcd3* that encodes Baf60c, a component of the BAF chromatin remodeling complex and a target of Wnt-signaling (Lickert et al., 2004). It will be important in the future to directly investigate the role of Wnt-signaling in the SHF and OFT myocardium.

The role of the Pitx2-mediated LRA pathway in OFT growth

At early stages, the SHF is correctly specified and SHF-derived cells move into the OFT of $Pitx2^{null}$ mutant embryos indicating that these processes are *Pitx2* independent. However, once the SHF populates the OFT, *Pitx2* is necessary for proper expansion of the proximal OFT myocardium. In the absence of *Pitx2* the OFT is shorter and misaligned. *Pitx2*-expressing cells are initially located on the left side of the OFT myocardium (Lickert et al., 2004; Liu et al., 2002; Takeuchi et al., 2005). Our data indicate that cell proliferation in the left OFT myocardium has a critical role in OFT lengthening.

However, it has also been shown that ablation of the right-sided SHF in chick embryos also resulted in a shortened OFT resulting in alignment defects and pulmonary atresia (Ward et al., 2005). These data indicate that regulation of myocardial expansion is not specific for the left-sided *Nodal–Lefty2–Pitx2* genetic pathway. Rather SHF cells on both sides of the embryo move into the OFT and contribute to OFT lengthening.

In addition to lengthening, the OFT undergoes a very characteristic spiraling motion (Ward et al., 2005). It may be that the *Pitx2*-mediated, left-specific pathway is important to direct

this spiraling morphogenesis. We previously suggested that *Pitx2* was not required for spiraling morphogenesis but in light of recent insights into OFT development this will have to be reevaluated in the future with higher resolution experiments (Liu et al., 2002). The possible role of *Pitx2* in spiraling morphogenesis cannot be rigorously evaluated in the $Pitx2^{null}$ embryos because of the shortened OFT. Nonetheless, our data provide a framework for future studies into the role of left–right patterning in development of the mature, functional OFT.

The role of Pitx2 in morphogenesis of the maturing OFT

Our findings suggest sequential activities for *Pitx2* in OFT development. The lineage tracing experiments presented here using the germline *Pitx2* mutant embryos indicate that *Pitx2* mutant daughter cells fail to efficiently populate the myocardium overlying the proximal aorta (Figs. 7A–D). This deficient contribution to the aortic myocardium may indicate that *Pitx2* regulates circumferential expansion of OFT myocardium. Recent lineage tracing experiments identified oriented circumferential cell growth in the OFT and RV myocardium (Meilhac et al., 2004b). However, it is also possible that *Pitx2* regulates local movement of OFT myocardium or OFT rotation rather than growth of the OFT myocardium (Bostrom and Hutchins, 1988; Lomonico et al., 1986). Indeed, recent findings using a novel transgene and Di-I labeling to mark the OFT myocardium have implicated *Pitx2c* in rotation of the OFT myocardium (Bajolle et al., 2006).

Interestingly, the chimera data indicate that $Pitx2^{null}$ mutant daughters are competent to contribute to the whole circumference of the proximal OFT. These data have two interpretations: *Pitx2* may regulate the expression of a signaling molecule that is required for contribution of *Pitx2*-daughters to the whole OFT myocardium. This model is plausible since it is known that *Pitx2* regulates the expression of signaling molecules in craniofacial development (Liu et al., 2003). It is also possible that in the germline mutant embryos, *Pitx2* mutant daughter cells inefficiently contribute to the aortic myocardium because of the early proliferative defect. In the context of a chimera, adequate numbers of *Pitx2* mutant daughter cells may escape the early proliferative deficiency and then expand to completely contribute to the proximal OFT. Further insight into these issues will require improved knowledge of *Pitx2* target genes.

We also found evidence that *Pitx2* autonomously regulated OFT remodeling. In both high and low percentage chimeras, we found that *Pitx2* mutant daughter cells accumulated at the base of the forming great vessels in the proximal OFT indicating a cell autonomous function for *Pitx2* in great vessel remodeling. It is known that apoptosis has an important role in OFT remodeling (Sugishita et al., 2004). It is possible that *Pitx2* may promote apoptosis at later stages of OFT development. Experiments are in progress to test this notion. Alternatively, it is possible that *Pitx2* regulates changes in cell shape in proximal OFT myocardium. Support for this comes from experiments performed in HeLa cells where overexpression of *Pitx2* enhanced cell spreading through regulation of small GTPase activity by transcriptional regulation of the *Trio* guanine nucleotide exchange factor (GEF) (Wei and Adelstein, 2002).

Non-canonical Wnt signaling in the OFT myocardium

The non-canonical Wnt-signaling pathway is β catenin independent but utilizes Dsh and Rho (Park and Moon, 2002). Previous work uncovered a strong genetic interaction between *Dsh2* and *Pitx2* in the OFT (Kioussi et al., 2002). In light of our findings presented here, one hypothesis is that *Pitx2* interacts with *Dsh2* as part of a non-canonical Wnt pathway. The *Loop-tail* (*Lp*) mouse mutant is a naturally occurring mouse mutant that was initially identified based on severe defects in neural tube closure (Phillips et al., 2005). The gene mutated in *Lp* is *Vangl2*, a homolog of the *Drosophila* planar cell polarity gene, *Strabismus*. Recent work has shown that *Vangl2* functions in the OFT myocardium to regulate OFT septation.

Oriented cell division has been observed in the OFT myocardium and, based on computer modeling, has been suggested to result from oriented mitosis (Meilhac et al., 2004a; Meilhac et al., 2004b). Non-canonical Wnt-signaling pathways are known to regulate convergent extension and cell intercalation, as well as oriented cell division in the gastrulating zebrafish embryo (Gong et al., 2004). It will be important in the future to investigate a connection between *Pitx2* and non-canonical Wnt signaling.

Insights into OFT development

Although substantial progress has been made, the mechanisms underlying OFT alignment, septation and separation into the aorta and pulmonary trunk remain poorly understood (Buckingham et al., 2005; Webb et al., 2003). The SHF functions as a source of myocardium for a rapidly expanding OFT. Because the OFT undergoes a spiraling morphogenetic movement, defects in the SHF or SHF-derived myocardium would be predicted to result in abnormal great vessel alignment such as DORV or TGA (Ward et al., 2005; Yutzey and Kirby, 2002). More recent experiments in chick embryos have shown that the SHF also makes a late contribution to smooth muscle cells of the great vessels (Waldo et al., 2005). The SHF also contributes cells to the OFT endocardium indicating that another function for the SHF may be to regulate OFT valve morphogenesis. Our findings extend these ideas by showing that *Pitx2*-dependent LRA signaling pathway is required to promote proliferation of a subset of OFT myocardium.

Molecular analysis has defined both *Isl1–Gata*- and *Foxh1–Nkx2.5*-pathways that regulate *Mef2c* expression in the SHF subpopulation of the second lineage (Dodou et al., 2004; von Both et al., 2004). Moreover, there is evidence that that *Tbx1* interacts with Fgf and *Pitx2*-mediated pathways to regulate SHF development (Hu et al., 2004; Nowotschin et al., 2006; Xu et al., 2004). Importantly, the *Isl1–Mef2c* pathway that is confined to the anterior-most region of the second lineage reveals that genetic regulation of the second lineage is subdivided into anterior and posterior fields. Our data reveal that the second lineage is also patterned along the left–right axis.

Implications for left–right asymmetry and cardiac development

Our data provide insight into the connection between early or embryonic LRA and cardiac morphogenesis. Previous models held that a linear signaling cascade asymmetrically patterned all developing organs (Levin and Mercola, 1998). In this framework, heterotaxia or

discordant organ morphogenesis was difficult to reconcile. To account for heterotaxia, the biasing model proposed that local asymmetry was an all or none, intrinsic property of each organ (Brown and Wolpert, 1990). Our data, showing that the second cardiac lineage is asymmetrically patterned by *Pitx2*, indicate that cardiac heterotaxia likely results from genetic defects in cell populations that contribute to the heart at distinct times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.ydbio.2006.06.009.

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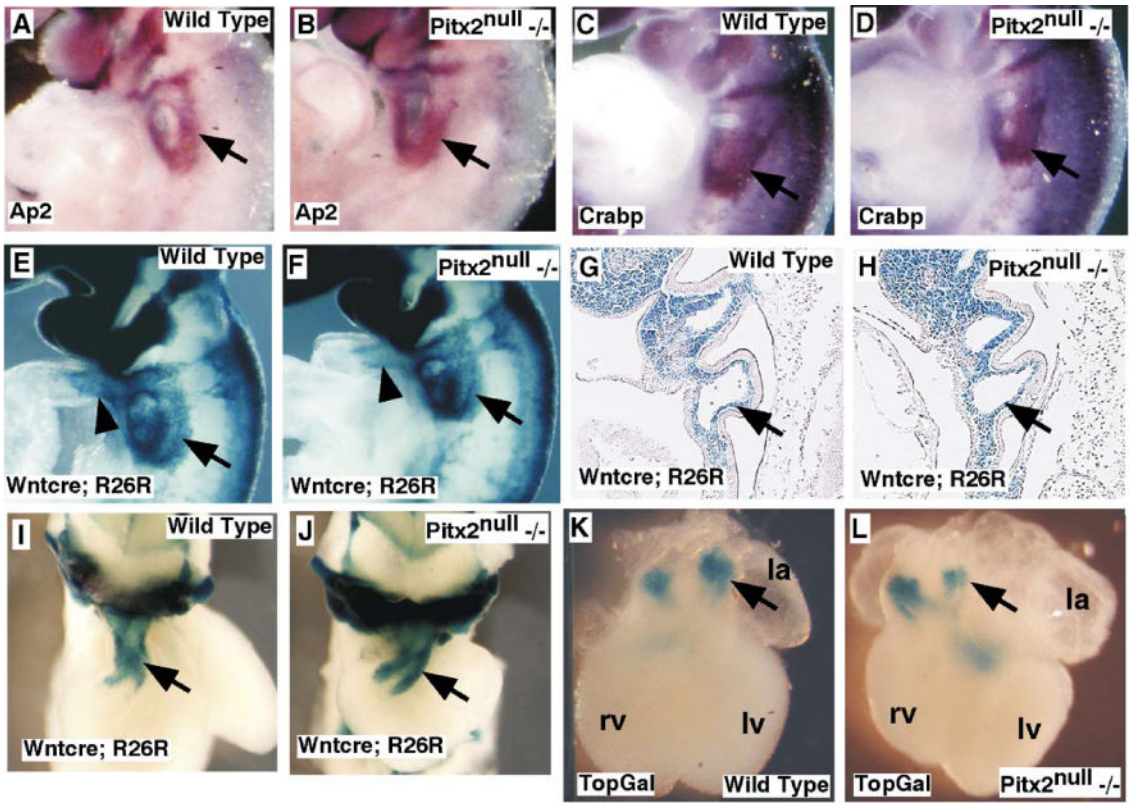

Fig. 1.

CNC marker analysis in *Pitx2*^{null} mutant embryos. In situ hybridization analysis with *Ap2* probe (A, B) and *Crabp* probe (C, D) on 9.5-dpc wild-type and *Pitx2*^{null} mutant embryos. Black arrows denote CNCs expression in branchial arch mesoderm and no obvious change between wild-type and Pitx2 mutant. (E–H) Left side view of whole-mount lacZ staining (E, F) and sagittal sections (G, H) of Wnt-1^{cre};R26R on 9.5-dpc wild-type (E, G) and 9.5-dpc Pitx2^{null} mutant embryos (F, H) indicating that CNC's migrate into the 3rd and 4th branchial arches (arrows denoted). Arrowheads denote the CNCs in the OFT. (I, J) Frontal view of whole-mount lacZ staining of Wnt-1^{cre};R26R on 9.5-dpc wild-type (I) and *Pitx2*^{null} mutant (J) to show CNC's migrate into outflow tract (arrows). TopGal activity in 12.5-dpc wild-type embryo (K) and *Pitx2*^{null} mutant (L). Arrows denote TopGal Activity. OFT, outflow tract; la, left atrium; lv, left ventricle; rv, right ventricle.

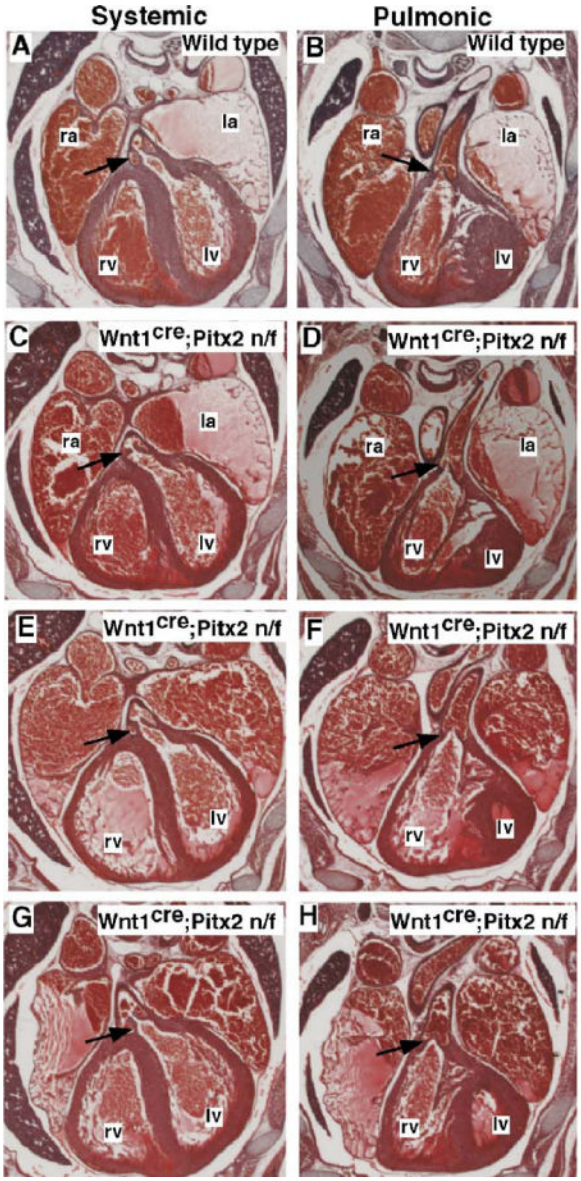

Fig. 2.

Conditional inactivation of *Pitx2* in the CNC. Serial coronal sections show systemic circulation of 14.5-dpc wild-type (A) and $Wnt1^{Cre}$;Pitx2 (n/f) heart (C, E, G). Arrows denote that the aortas connect to the left ventricle. Serial coronal sections show pulmonic circulation of 14.5-dpc wild-type (B) and $Wnt1^{Cre}$;Pitx2 (n/f) heart (D, F, H). Arrows denote the right connection between pulmonary arteries and right ventricles. la, left atrium; ra, right atrium; lv, left ventricle; rv, right ventricle.

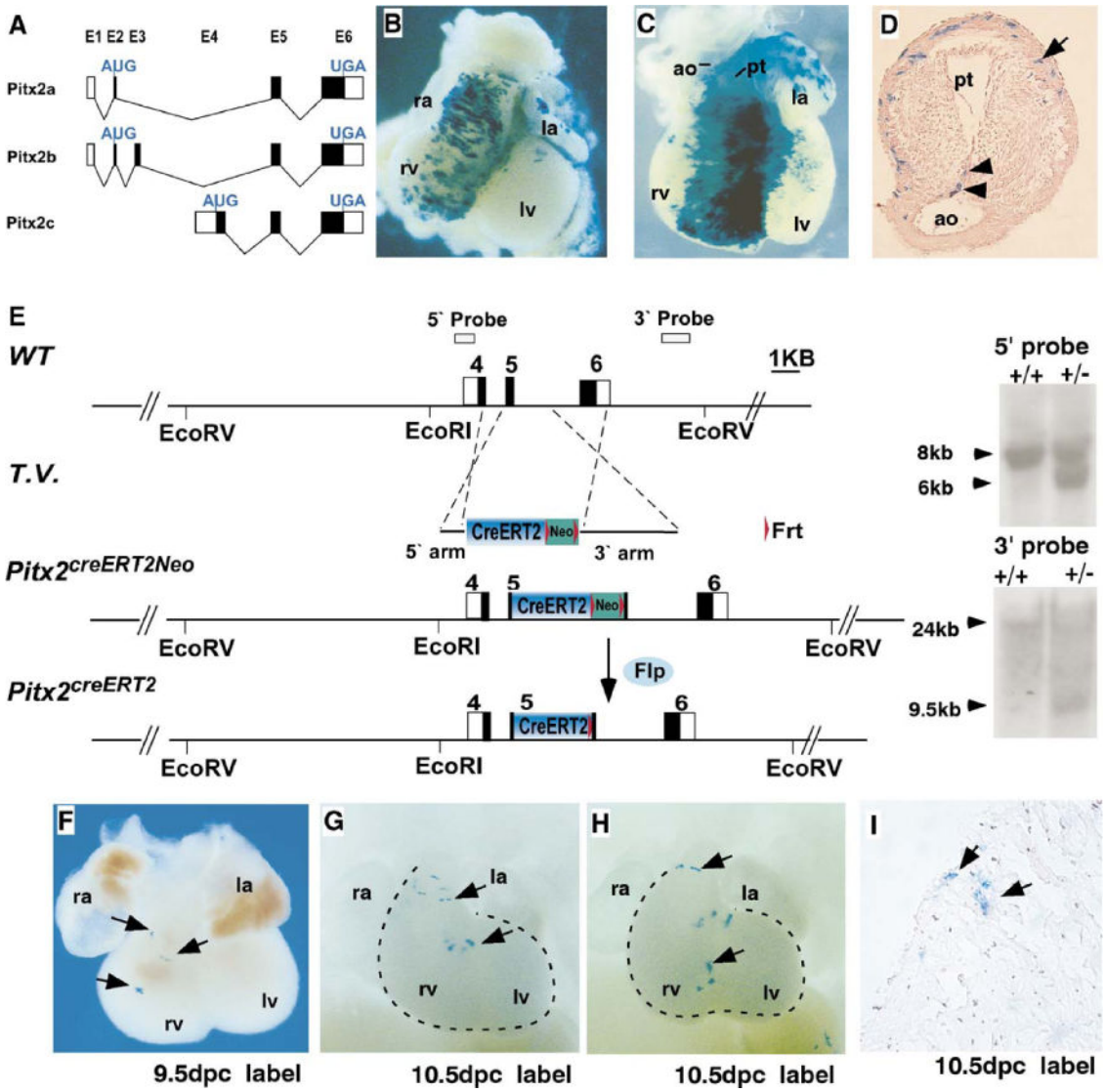

Fig. 3.

Pitx2^{cre} lineage analysis and *Pitx2*^{creERT2} fate mapping. (A) Summary of exon usage by *Pitx2* isoforms. (B, C) Frontal view of LacZ staining of a dissected 11.5-dpc (B) and 14.5 dpc (C) *Pitx2*^{cre}*;R26R* hearts and a transverse section through the 11.5 dpc OFT (D). *Pitx2* daughters denoted by an arrow (OFT myocardium) and arrowheads (endocardium). (E) *Pitx2* genomic structure, *Pitx2*^{creERT2} knock-in targeting strategy, and targeted clones confirmed by southern blot. Boxes represent exons and straight lines, introns. P1 and P2 indicate two promoters that initiate transcription of different isoforms. (F–H) LacZ staining of 12.5-dpc *Pitx2* ^{creERT2}; *R26R* hearts, labeled at 9.5 dpc and 10.5 dpc. (I) Transverse section of OFT in panel G. Arrows denote *Pitx2* daughter cells. la, left atrium; ra, right atrium; lv, left ventricle; rv, right ventricle.

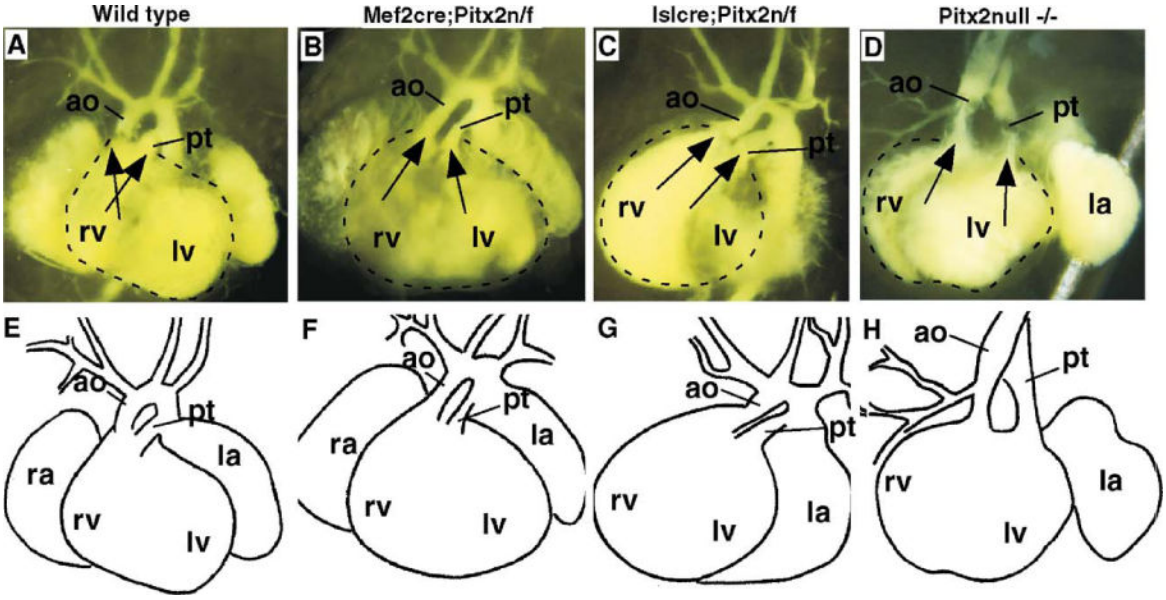

Fig. 4.

Conditional inactivation of *Pitx2* in the SHF. Dye injection into 14.5-dpc hearts to show OFT morphology with schematic diagram below. (A, E) Wild-type, (B, F) $Mef2c^{cre};Pitx2^{n/f}$ showing TGA, (C, G) $Isl1^{cre};Pitx2^{n/f}$ showing DORV, (D, H) $Pitx2^{null}$ mutant showing TGA. Note that each diagram is associated with its above picture. Arrows denote vessel alignment. TGA, transposition of great artery; DORV, double outlet of right ventricle; ao, aorta; pt, pulmonary trunk; la, left atrium; ra, right atrium; lv, left ventricle; rv, right ventricle.

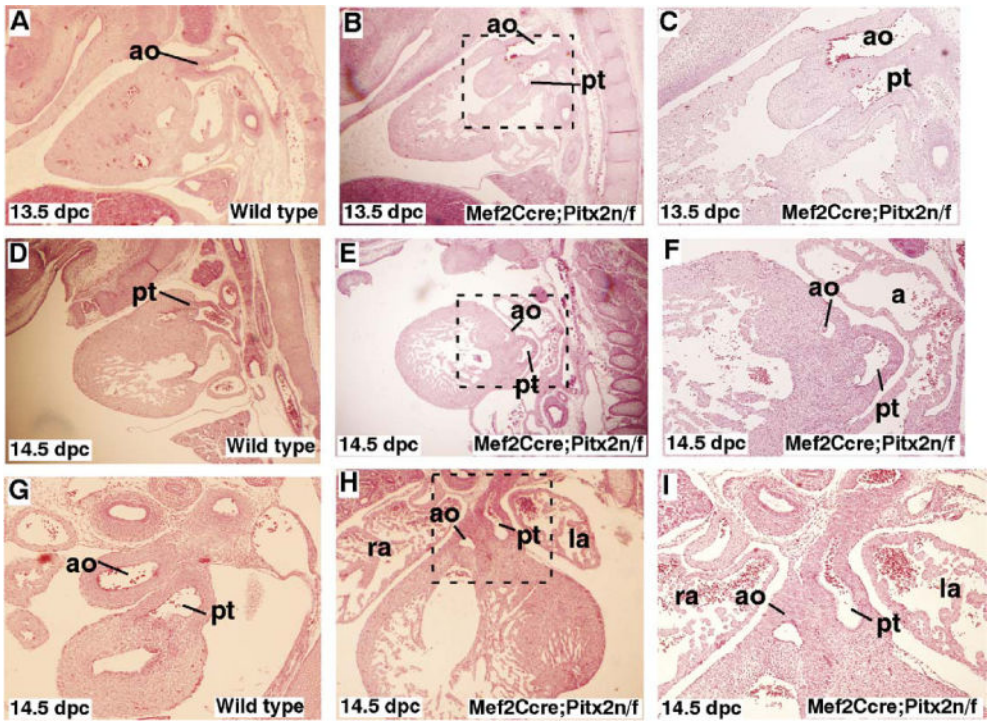

Fig. 5.

Sections of *Pitx2* SHF deficient embryos. Sagittal sections through 13.5-dpc (A–C), and 14.5-dpc (D–F) embryos. (C, F) Higher magnification views of boxed areas in panels B and E. (G–I) Modified transverse sections of 14.5-dpc $Mef2c^{cre}; Pitx2^{n/f}$ heart showing abnormal arterioventricular alignment. (I) Higher magnification image of boxed area in panel H. Genotypes and stages are labeled. ao, aorta; pt, pulmonary trunk; la, left atrium; ra, right atrium.

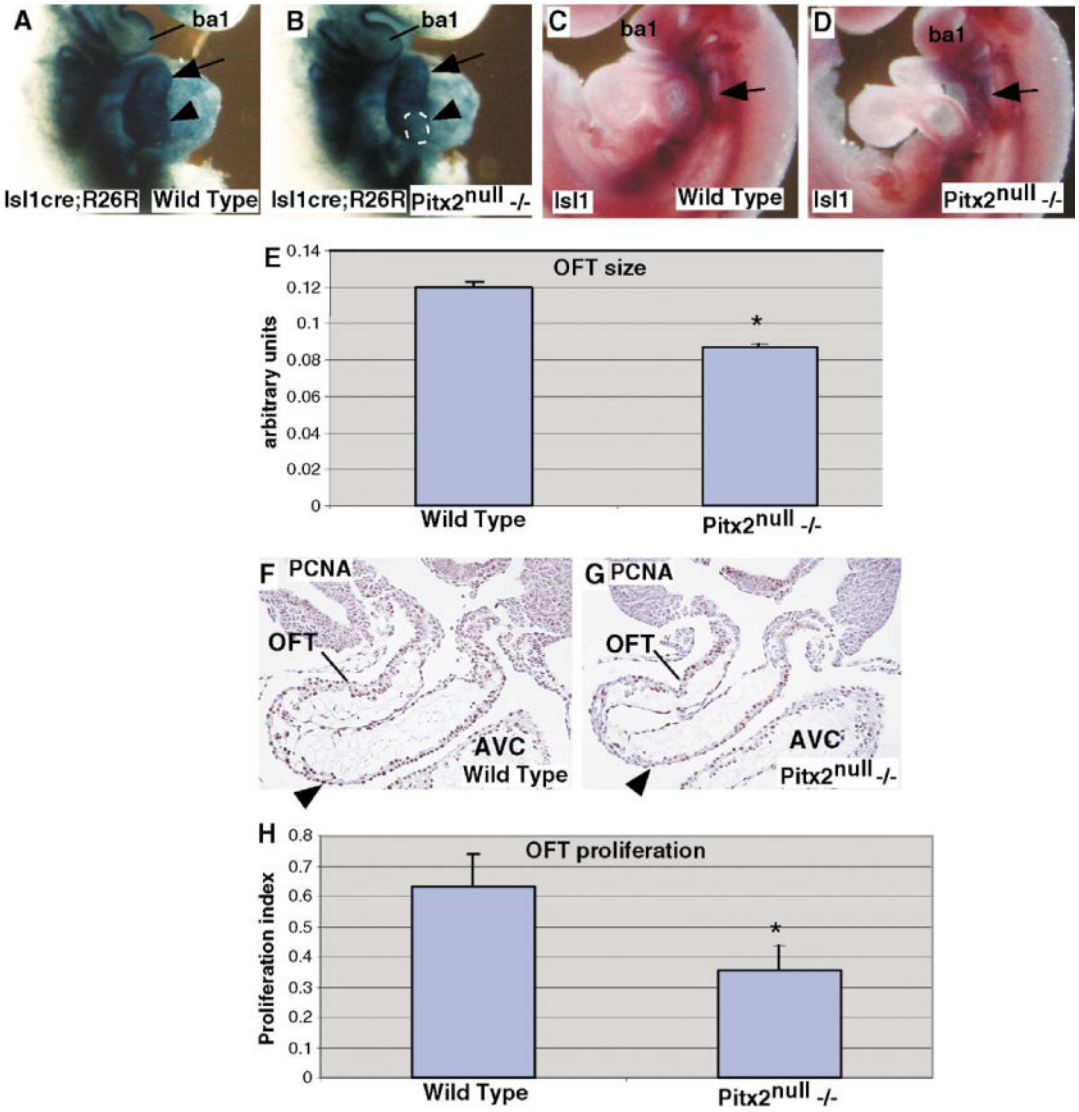

Fig. 6.

Secondary heart field marker analysis in *Pitx2* null mutants. Right lateral view of *Isl1* fate mapping in 9.5 dpc control (A) and *Pitx2*^{null} mutant embryo (B). Arrow and arrowhead denote distal and proximal OFT respectively. Circled area in panel B denotes the area of reduced LacZ staining. (C, D) Left views of whole-mount in situ hybridization with *Isl1* probe in 9.5 dpc control (C) and *Pitx2* mutant embryo (D). Arrows denote branchial arch mesoderm. (E) Bar graph quantitation of OFT size based on morphometric analysis as described in Materials and methods. (*$P < 0.02$, Student's *t*-test). (F, G) PCNA assay shows that cell proliferation is reduced in *Pitx2* mutant proximal OFT compared to control. Arrowheads denote proximal outflow tract. (H) Bar graph of proliferation Index is shown in panel H. (*$P < 0.01$, Student's *t*-test). ba1, first branchial arch; OFT, outflow tract; AVC, atrioventricular canal.

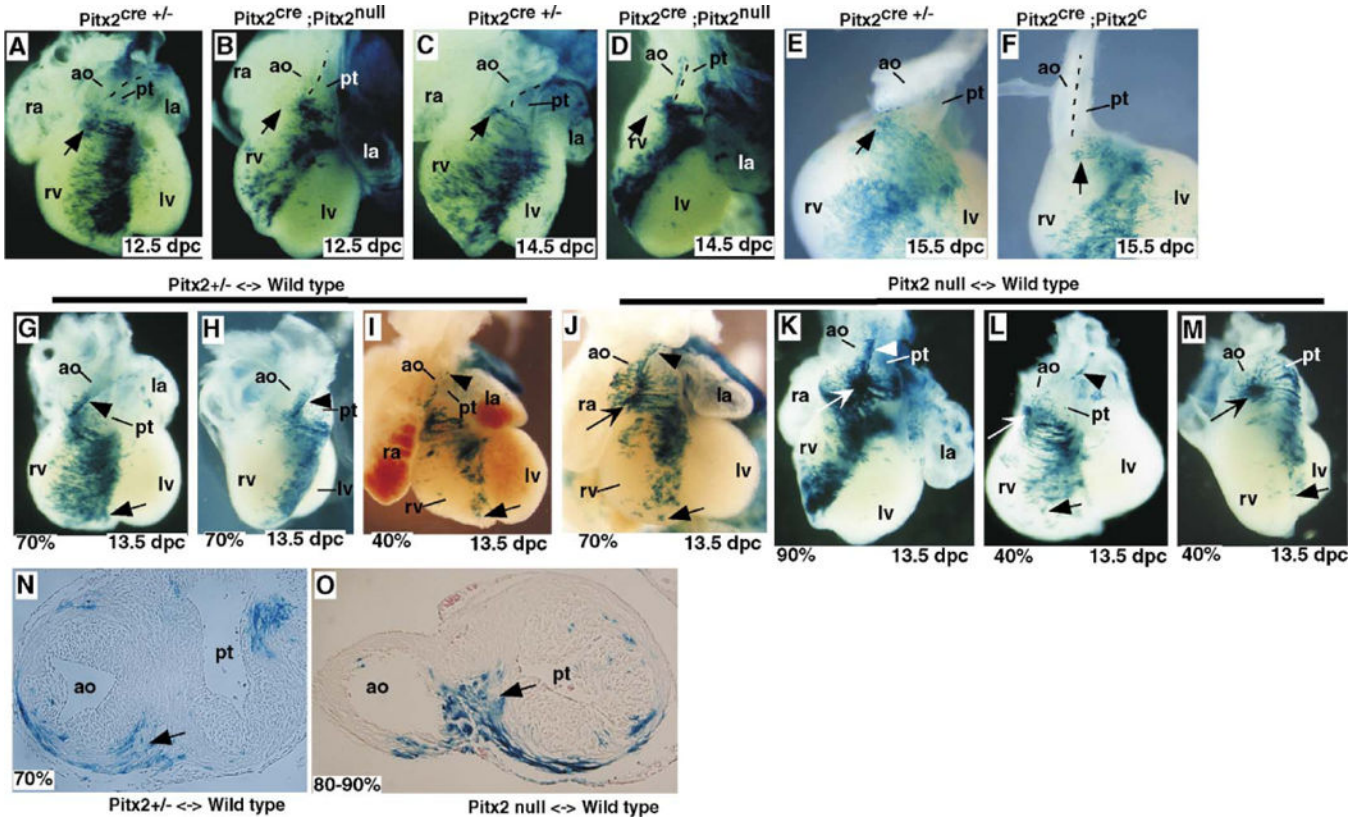

Fig. 7.

Chimera analysis of *Pitx2* function in the OFT. (A, B) Lineage tracing with the *Pitx2*^{cre} and *R26R* alleles in control (A, C, E) and *Pitx2* mutant embryos (B, D, F). *Pitx2* daughter cells are stained with LacZ. Arrows denote the *Pitx2* daughters that are normally contribute to proximal aortic myocardium and are deficient in the mutant embryos. Note that the *Pitx2c* allele is a deletion of the *Pitx2c* isoform that allows for analysis of later stage embryos. (G–I) *Pitx2*+/− ↔wild type chimeras at 13.5 dpc. Percentage chimerism is labeled and *Pitx2* daughters in the OFT denoted by arrowhead and in RV by the arrows. (J–M) *Pitx2*−/− ↔wild type chimeras at 13.5 pc. Percentage chimerism is shown. *Pitx2* daughters in the OFT denoted by arrowhead and in RV by the arrows. Angled arrows indicate the *Pitx2* mutant daughters that abnormally persist at the junction of the aorta and pulmonary trunk. (N, O) Sections through the OFT of *Pitx2*+/− ↔ wild type chimeras (N) and *Pitx2*−/− ↔ wild type chimera (O). Arrows indicate cells that abnormally persist in the mutant chimera. ao, aorta; pt, pulmonary trunk; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.