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DEVELOPMENT AND DISEASE

Pitx2c patterns anterior myocardium and aortic arch vessels and is required for local cell movement into atrioventricular cushions

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SUMMARY

Inactivation of the left-right asymmetry gene *Pitx2* has been shown, in mice, to result in right isomerism with associated defects that are similar to that found in humans. We show that the *Pitx2c* isoform is expressed asymmetrically in a presumptive secondary heart field within the branchial arch and splanchnic mesoderm that contributes to the aortic sac and conotruncal myocardium. *Pitx2c* was expressed in left aortic sac mesothelium and in left splanchnic and branchial arch mesoderm near the junction of the aortic sac and branchial arch arteries. Mice with an isoform-specific deletion of *Pitx2c* had defects in asymmetric remodeling of the aortic arch vessels. Fate-mapping studies using a *Pitx2 cre recombinase* knock-in allele showed that daughters of *Pitx2*-expressing cells populated the right and left ventricles, atrioventricular cushions and valves and pulmonary veins. In *Pitx2* mutant embryos, descendents of *Pitx2*-expressing cells failed to contribute to the atrioventricular cushions and valves and the pulmonary vein, resulting in abnormal morphogenesis of these structures. Our data provide functional evidence that the presumptive secondary heart field, derived from branchial arch and splanchnic mesoderm, patterns the forming outflow tract and reveal a role for *Pitx2c* in aortic arch remodeling. Moreover, our findings suggest that a major function of the *Pitx2*-mediated left right asymmetry pathway is to pattern the aortic arches, outflow tract and atrioventricular valves and cushions.

Key words: Cardiac development, Homeobox, Morphogenesis, Mouse

INTRODUCTION

Pitx2 is a paired-related homeobox gene that has been shown to play a central role in the late aspects of left-right asymmetric morphogenesis (Capdevila et al., 2000; Harvey, 1998). Identified as the gene mutated in Rieger syndrome 1, *Pitx2* also functions in eye, tooth and abdominal wall development (Alward, 2000; Semina et al., 1996). Importantly, *Pitx2* has been shown to be a direct target of the left-right signaling pathways that originate early in development through the function of nodal (Shiratori et al., 2001). Loss-of-function experiments performed in mice have revealed a role for *Pitx2* in left-right asymmetry of many organs but its role in heart and vascular development is less clear (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999b).

Although *Pitx2*-null mice have severe cardiac phenotypes that are similar to those observed in humans with laterality defects, the *Pitx2*-null phenotype suggests that *Pitx2* function is important after looping morphogenesis, as *Pitx2* mutant hearts loop correctly to the right (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Liu et al., 2001; Lu et al., 1999b). Analysis of individuals with laterality defects has revealed a spectrum of associated cardiac septation and valve anomalies, including abnormalities in conotruncal and right ventricular development, atrial lateralization and atrioventricular (AV) septation (Brown and Anderson, 1999; Icardo and Sanchez de Vega, 1991). These observations suggest that the genetic pathways regulating left-right asymmetry may also directly regulate valve and cushion morphogenesis and that subtle defects in left-right asymmetry may be a common etiologic factor for congenital heart disease.

In common with human patients, *Pitx2*-null mice display atrial septal defects (ASD), abnormal AV septation (resulting in complete AV canal) and abnormal arterioventricular connections (Kitamura et al., 1999; Liu et al., 2001). *Pitx2* mutants also have a hypoplastic right ventricle. Although *Pitx2* mutant mice have severe cardiac anomalies, the primary function of *Pitx2* in heart development remains unclear as the *Pitx2* mutant heart phenotypes could be secondary to delayed looping morphogenesis or embryonic rotation.

In this work, we have used a combination of gene expression analysis and gene targeting approaches to investigate *Pitx2* function in cardiovascular development in more detail. Our data demonstrate that the *Pitx2c* isoform is expressed in a presumptive secondary heart field that invades the heart after looping morphogenesis. *Pitx2c* was expressed in a subpopulation of left branchial arch and splanchnic mesoderm apposed to forming branchial arch arteries (BAAs) and in left aortic sac mesothelium. An isoform-specific deletion of *Pitx2c*, generated by gene targeting in embryonic stem cells, revealed that *Pitx2c* functions to regulate asymmetric BAA remodeling and to pattern the outflow tract (OFT). Fate-mapping studies with a *Pitx2 cre* knock-in allele revealed that *Pitx2* daughter cells invade the AV cushions and valves in a *Pitx2*-dependent fashion, suggesting a role for *Pitx2* in local cell movement or survival within the heart. Our results provide insight into *Pitx2* function in post-looping cardiac morphogenesis and in BAA remodeling.

MATERIALS AND METHODS

Gene targeting in ES cells

To generate the *Pitx2 δc neo* targeting vector, we replaced the *Pitx2* exon 4 that encodes the *Pitx2c*-specific exon with a LoxP flanked PGKneomycin cassette. The *Pitx2 δc neo* targeting vector introduced a novel *Eco*RV site into the mutant *Pitx2* locus that we used to screen for homologous recombination events by Southern blot using flanking probes.

After homologous recombination, the *Pitx2 δc neo* allele resulted in deletion of the majority of exon 4, including all coding sequences within this exon. The *Pitx2 δc neo* targeting vector was electroporated into AK7 ES cells, targeted clones identified by Southern blot, and injected into 3.5 dpc C57BL/6J mouse embryos to generate chimeras. To induce recombination between the two loxP sites and remove PGKneomycin cassette, we crossed *Pitx2 δc neo* chimeras to CMVCre recombinase deleter strain. *Pitx2 δc neo* and *δc* alleles were maintained on a mixed 129/Sv×C57BL/6J genetic background.

The *Pitx2 δabc*^{creneo} will be described elsewhere. Briefly, to generate this allele, an IRES cre PGKneomycin cassette was introduced into the *Pvu*II and *Nru*I sites of *Pitx2* exon 5, that encodes part of the homeodomain, generating a null allele of *Pitx2*. Whole-mount in situ with a *cre recombinase* (*cre*) probe confirmed that expression of *cre* recapitulated endogenous *Pitx2* expression pattern.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Lu et al., 1999b).The *Pitx2c* probe was a 1 kb genomic fragment containing exon 4 that was linearized with *Xho*I and transcribed with T7 polymerase. The semaphorin 3c probe has been described previously (Brown et al., 2001) and the *cre* probe was a cDNA fragment that was linearized with *Eco*RI and transcribed with T7 polymerase.

lacZ staining and histology

For histology, embryos were fixed overnight in buffered formalin, dehydrated through graded ethanol and paraffin embedded. Sections were cut at 7-10 μm and H&E stained. *lacZ* staining was described (Lu et al., 1999a).

Corrosion cast and casting dye injections

Injection of casting dye: 18.5 dpc embryos were harvested and sternum removed. Yellow casting dyes (Connecticut Valley Biological Supply) were injected into right ventricles using a capillary pipette, followed by blue dye into left ventricle. Corrosion casts: 18.5 dpc embryos were isolated and the heart exposed by a thoracic incision. Batson number 17 acrylic (Polysciences) was injected into right and left ventricles until great arteries were filled. After hardening overnight in distilled water at 4°C, tissues were removed with Maceration Solution at 50°C for 24 hours without shaking.

India Ink Injections

Embryos were dissected and placed in ice cold PBS. Individual embryos were placed in warm PBS to facilitate ventricular contractions. Using a pulled glass pipette, India Ink was injected into ventricles until ink penetrated small vessels. Embryos were post fixed in 10% formalin and cleared in benzyl alcohol:benzyl benzoate (2:1).

RESULTS

Pitx2c is asymmetrically expressed in splanchnic and branchial arch mesoderm and outflow tract myocardium

Previous studies have shown that the *Pitx2c* isoform is asymmetrically expressed in the developing embryo while the *Pitx2a* and *Pitx2b* isoforms are co-expressed with *Pitx2c* in symmetrical regions of the embryo (Kitamura et al., 1999; Liu et al., 2001; Schweickert et al., 2000; Yu et al., 2001). For example, *Pitx2c* is expressed in left lateral plate mesoderm and in the left side of most organ primordial such as developing guts, heart and lungs. The three *Pitx2* isoforms in mice are co-expressed in periocular mesenchyme, oral and dental epithelium, as well as anterior body wall. A fourth *Pitx2* isoform, *Pitx2d*, has recently been described in humans (Cox et al., 2002).

Because of the correct dextral looping in *Pitx2* null embryos, we hypothesized that *Pitx2* functioned in a cell population that contributed to the heart during or after cardiac looping. Experiments performed in chick and mouse embryos have revealed that cells outside the primary heart field contribute to conotruncal development. One cell population originates in the splanchnic and branchial arch mesoderm, and migrates into the OFT and right ventricle of the looped heart (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). We found that cells within this presumptive secondary heart field express *Pitx2c* asymmetrically at 9.5 dpc both as they migrate and after populating the OFT and right ventricle, revealing that this cell population has laterality.

Whole-mount in situ using a *Pitx2c*-specific probe on 9.5 dpc embryos revealed left-sided *Pitx2c* expression in splanchnic mesoderm at the level of and just caudal to the OFT (Fig. 1A,B). Serial sectioning showed that *Pitx2c* expression in left splanchnic mesoderm was continuous with expression in left aortic sac mesothelium and OFT myocardium (Fig. 1C-F). Expression of the *Pitx2a* and *Pitx2b* isoforms was not detected in the presumptive secondary heart field or developing OFT (not shown).

We examined coronal and parasagittal sections through 9.5 and 10.5 dpc embryos to investigate in more detail the *Pitx2c* expression pattern during these timepoints prior to remodeling of the BAA. Ventral coronal sections through 9.5 dpc embryos showed left-sided *Pitx2c* expression in aortic sac mesothelium near the junction of the aortic sac and the BAA (Fig. 1F). More dorsal coronal sections revealed low levels of *Pitx2c* expression in left branchial arch and splanchnic mesoderm in proximity to the third BAA (Fig. 1G). Serial parasagittal sections through 10.5 dpc embryos showed *Pitx2c* expression

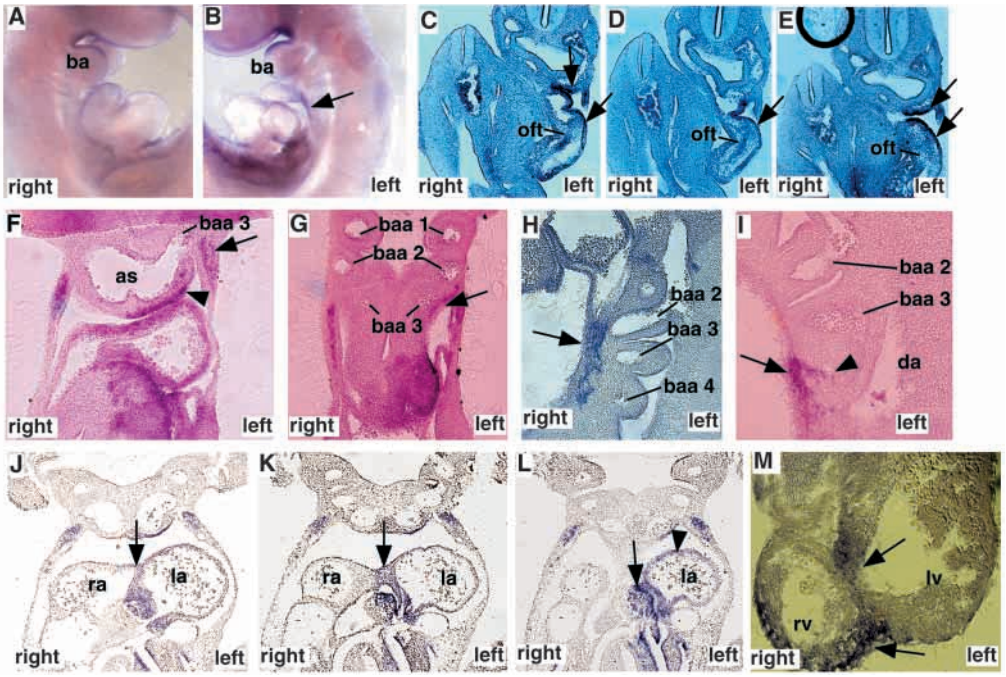

Fig. 1. Expression of *Pitx2c* in the branchial arch mesoderm and aortic sac. (A,B) Whole-mount images of 9.5 dpc wild-type embryos hybridized to a *Pitx2c*-specific probe showing *Pitx2c* in left branchial arch mesoderm (arrow in B). (C-E) Transverse serial sections through a 9.5 dpc wild-type embryo hybridized to *Pitx2c* probe. *Pitx2c* expression is denoted by arrows. (F,G) Coronal sections through a 9.5 dpc embryo hybridized to a *Pitx2c* probe showing *Pitx2c* expression in left branchial arch mesoderm near junction of the aortic sac with the branchial arch artery (arrow in F). *Pitx2c* expression within left aortic sac myocardium is denoted by arrowhead. In G, arrow designates *Pitx2c* expression in dorsal branchial arch mesoderm in proximity to forming branchial arch arteries. (H,I) Parasagittal section through a10.5 dpc embryo hybridized to *Pitx2c* probe. Arrow denotes ventral *Pitx2c* expression while arrowhead in I indicates less abundant dorsal *Pitx2c* expression. (J-M) Transverse sections through hearts of 10.5 dpc embryos hybridized to *Pitx2c* probe. Arrows in J,K,L indicate *Pitx2c* expression in dorsal mesocardium, while arrowhead in L indicates *Pitx2c* expression in left atrium. Arrows in M indicate *Pitx2c* expression in right ventricular and interventricular myocardium. as, aortic sac; ba, branchial arch; baa, branchial arch artery; da, dorsal aorta; oft, outflow tract.

in ventral branchial arch and splanchnic mesoderm that was continuous with *Pitx2c* expression in left branchial arch mesoderm evident on more lateral sections (Fig. 1H,I). The parasagittal sections at this timepoint also revealed the diminished intensity in *Pitx2c* expression dorsally towards the dorsal aorta (Fig. 1I). Our expression studies also showed *Pitx2c* expression in the left atrium, primary interatrial septum, left dorsal mesocardium, and right ventricular and interventricular myocardium (Fig. 1J-M). From these studies, we conclude that *Pitx2c* is asymmetrically expressed in a subpopulation of the presumptive secondary heart field that contributes to the OFT and right ventricular myocardium after cardiac looping, suggesting that *Pitx2c* provides laterality to the OFT and right ventricle myocardium. Moreover, asymmetric *Pitx2c* expression in ventral branchial arch and splanchnic mesoderm, with higher levels ventrally near the junction of the BAA and aortic sac, suggests a role for *Pitx2c* in formation of the BAAs.

Pitx2c mutants survive gestation and turn normally

The *Pitx2c* isoform is encoded by exons 4, 5 and 6, and uses a distinct promoter from that of the *Pitx2a* and *Pitx2b* isoforms (Shiratori et al., 2001). The *Pitx2* exon 5 and exon 6, which encode the homeodomain, are common to all *Pitx2* isoforms in mice (Fig. 2A,B). To directly investigate *Pitx2c* function using a loss-of-function approach in mice, we constructed a targeting vector that replaced the *Pitx2c*-specific exon 4 with a PGKneomycin LoxP cassette, the *Pitx2 δc* neo targeting vector (Fig. 2B,C). Upon germline transmission, the *Pitx2 δc neo* allele was crossed to the *cmv cre recombinase* deletor strain to remove the *PGK neomycin* cassette and generate the final *Pitx2 δc* allele (Fig. 2C,D). Both *Pitx2 δc neo* and *Pitx2 δc*$^{-/-}$ mutants were obtained at the Mendelian ratio at 18.5 dpc. Mutant neonates were born alive but quickly became cyanotic and died a few minutes after birth. We noted that *Pitx2 δc*$^{-/-}$ mutants turned normally, suggesting that the *Pitx2a* and *Pitx2b* isoforms have redundant function with *Pitx2c* in turning or body wall closure as *Pitx2a,Pitx2b* homozygous mutant embryos also turn normally (Liu et al., 2001) (Fig. 2E).

Pitx2c patterns the aortic arch vessels

To determine if *Pitx2c* had a role in patterning the great vessels of the aortic arch, we performed casting dye and corrosion cast experiments on 18.5 dpc *Pitx2 δc*$^{-/-}$ embryos. Among the 21 mutant embryos examined, 57% (12 out of 21) had the wild-type pattern of left aortic arch with right innominate artery while 29% (six out of 21) had right aortic arch with left innominate artery (Fig. 3A-D). In addition, 14% (three of 21) showed double aortic arch without innominate artery (Fig. 3E-J). Of the double aortic arches, two were right dominant and the other left dominant. Thus, of all arches examined, 62% (n=13) were left dominant and the remaining 38% (n=6) were right dominant. In addition, all *Pitx2 δc*$^{-/-}$ embryos had double outlet right ventricle (DORV) in which both the aorta and pulmonary artery drain the right ventricle (Fig. 3O,P). Blood exited the left ventricle of the *Pitx2 δc*$^{-/-}$ embryos through a ventricular septal defect.

The corrosion casting experiments revealed that *Pitx2c* had an important role in patterning of the BAAs. To determine if *Pitx2c* had a role in the initial formation of the BAAs or was important in BAA remodeling, we performed India ink injections at 11.0 and 11.5 dpc at the initiation of BAA remodeling. At these timepoints, all *Pitx2c* mutant embryos (n=6) formed symmetric BAAs that were indistinguishable

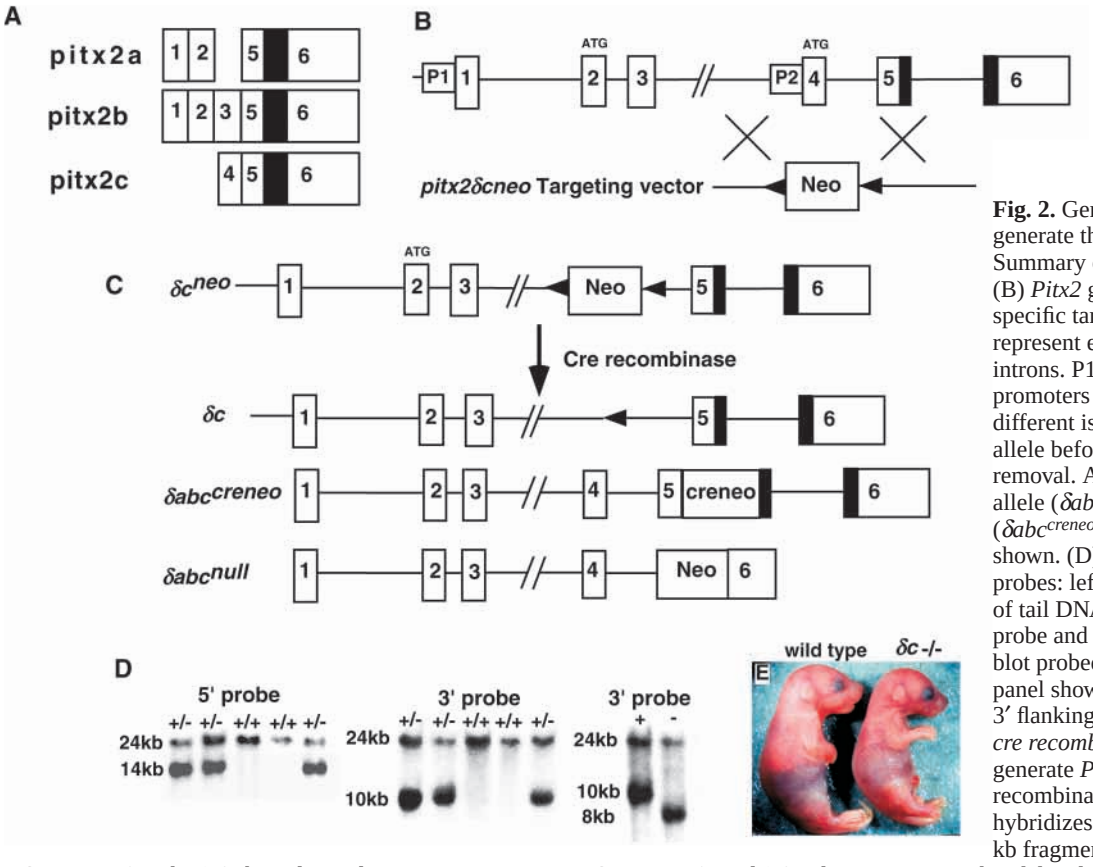

Fig. 2. Gene targeting strategy to generate the *Pitx2 δc* allele. (A) Summary of exon use by *Pitx2* isoforms. (B) *Pitx2* genomic structure and *Pitx2c*-specific targeting strategy. Boxes represent exons and straight lines introns. P1 and P2 indicate two promoters that regulate expression of different isoforms. (C) *Pitx2 δc* targeted allele before and after *PGKneomycin* removal. At the bottom, the *Pitx2* null allele (δabc^{null}) and cre knock-in alleles (δabc^{creneo}) also used in this study are shown. (D) Southern blot with flanking probes: left panel shows a Southern blot of tail DNA probed with 5′ flanking probe and center panel shows Southern blot probed with 3′ flanking probe. Right panel shows a Southern blot probed with 3′ flanking probe after crossing to *CMV cre recombinase* deletor strain to generate *Pitx2* $\delta c^{+/-}$ mice. After recombination, the 3′ flanking probe hybridizes to an 8 kb fragment and a 10 kb fragment in mice retaining *PGKneomycin*. The '+' above lanes denotes mice retaining *PGKneomycin* and '–' indicates a mouse that deleted *PGKneomycin.* (E) Lateral view of wild-type and *Pitx2* $\delta c^{-/-}$ 18.5 dpc embryos.

from wild type littermates (Fig. 3K-N). From this, we conclude that *Pitx2c* functions in remodeling of the BAA. The very discrete, asymmetric *Pitx2c* expression pattern within the region of the forming BAAs also supports the idea that *Pitx2c* would have a role in modulating BAA remodeling rather than in the initial endothelial tube assembly.

Cardiac neural crest migrates normally in *Pitx2c* mutants

Great vessel remodeling and patterning of the conotruncal region, both defective in *Pitx2c* mutants, require normal development of the cardiac neural crest. To determine if cardiac neural crest contributed to the conotruncal region of *Pitx2* $\delta c^{-/-}$ embryos, we performed a fate-mapping experiment with the *wnt1 cre* transgenic line that directed *cre* expression to the precursors of the cardiac neural crest and the *Rosa26* reporter line (Jiang et al., 2000; Soriano, 1999). *cre* expression will induce recombination at the *Rosa26* locus resulting in expression of *lacZ* in all descendents of *Wnt1*-expressing cells that include the cardiac neural crest. At both 11.5 and 12.5 dpc, we found that cardiac neural crest contributed normally to the conotruncal region and aortic and pulmonic valves of *Pitx2* $\delta c^{-/-}$ embryos suggesting that *Pitx2* function in conotruncal cushion morphogenesis occurred subsequent to neural crest migration into the *Pitx2* mutant heart (Fig. 4A-F). Analysis of sections through the branchial arch arteries of 10.5 dpc wild type and *Pitx2* $\delta c^{-/-}$ embryos showed similar amounts of mesenchyme surrounding the arteries further supporting the idea that cardiac neural crest was correctly deployed in *Pitx2* $\delta c^{-/-}$ embryos (Fig. 4G-J).

To determine how loss of *Pitx2* affected development of OFT myocardium, we examined the expression of semaphorin 3c (*Sema3c*), an OFT myocardial marker, in wild type and *Pitx2c* mutants (Brown et al., 2001; Feiner et al., 2001). Although at 10.5 and 11.5 dpc, *Sema3c* was expressed normally in *Pitx2c* mutant OFT myocardium (Fig. 5A-D), this expression was downregulated by 12.5 dpc (Fig. 5E,F). These data suggested that OFT myocardium was correctly specified and that migration of OFT myocardial precursors was intact in *Pitx2c* mutants.

To establish this more firmly, we used a *Pitx2* cre knock-in allele (*Pitx2* δabc^{creneo}), an allele of *Pitx2* that expresses *cre* in the endogenous *Pitx2* expression domain, to mark cells fated to express *Pitx2*. The *Pitx2* δabc^{creneo} allele has a *cre recombinasePGKneomycin* cassette introduced into *Pitx2* exon 5 to generate a null *Pitx2* allele, removing function of all *Pitx2* isoforms (see Materials and Methods, and Fig. 5G,H). Moreover, expression of *cre* from the *Pitx2* δabc^{creneo} qualitatively recapitulates the endogenous *Pitx2* spatiotemporal expression pattern (not shown). At both 10.5 and 12.5 dpc, spatial expression of *cre* was similar in the OFT of the control δabc^{creneo} heterozygotes and $\delta abc^{creneo};\delta abc^{null}$ *Pitx2*-null mutant embryos, supporting the idea that *Pitx2* patterns the OFT myocardium after it is established (Fig. 5I,J and not shown). Taken together, these data support the notion that *Pitx2* functions in branchial arch and splanchnic

mesoderm, a developmental field that is distinct from cardiac neural crest. Moreover, downregulation of *Sema3c* expression in *Pitx2* mutants suggests that *Pitx2* has a role in maintenance of gene expression in OFT myocardium.

Pitx2 daughter cells contribute to OFT, inner curvature myocardium and valves

In addition to cardiac neural crest, OFT and inner curvature myocardium invades the cardiac cushions (van den Hoff et al., 1999; van den Hoff et al., 2001). To determine if descendents of *Pitx2*-expressing myocardium populated the cardiac cushions, we used the *Pitx2* δabc^{creneo} allele and the *Rosa26* reporter allele to follow the fate of *Pitx2* daughter cells after *Pitx2* expression had been extinguished. At timepoints when *Pitx2c* is actively expressed in the heart, 9.5 dpc until 12.5 dpc, distinctions between *Pitx2* daughter cells and newly labeled *Pitx2*-expressing cells can be made in regions of the heart that never express *Pitx2c*. For example, at 9.5 and 10.5 dpc *Pitx2*-expressing cells are restricted to the left side of the forming OFT (Fig. 1C-E). By contrast, *lacZ*-positive cells were detected on both sides of the OFT tract myocardium, suggesting that labeled *Pitx2* daughter cells, found on the right side of the OFT, had moved from the left side (Fig. 6A). The distribution of *lacZ*-positive cells in the OFT tract in *Pitx2* null embryos was similar to that of the wild type, suggesting that *Pitx2* is not required for movement of the myocardial precursors from branchial arch mesoderm into the OFT (Fig. 6A,B). We noted that the number of *lacZ*-labeled cells in the OFT myocardium of 10.5 dpc embryos was less than what would be expected from the *Pitx2c* expression pattern. This may reflect the delay between *cre* transcription and Cre-mediated excision that requires the accumulation of adequate levels of Cre protein. Moreover, the delay in the readout is also lengthened by the need for transcription and translation of *lacZ* from the *Rosa26* locus (Nagy, 2000).

At 12.5 dpc, when *Pitx2c* is still expressed in the heart, the *Pitx2* δabc^{creneo} allele cannot distinguish between newly labeled *lacZ*-positive cells and *Pitx2c* descendents that are no longer expressing *Pitx2c*. At this timepoint, *lacZ*-labeled cells were predominantly found in the myocardium overlying the interventricular groove with some cells found in the proximal OFT (Fig. 6C,D). By 14.5 dpc and 16.5 dpc, when *Pitx2c* expression is extinguished (Fig. 6F,H), there was an increase of *lacZ*-positive *Pitx2c* descendents over the medial aspect of the heart, suggesting an outward expansion of *Pitx2c* descendents from the right ventricular and inner curvature myocardium (Fig. 6E,G). Sections

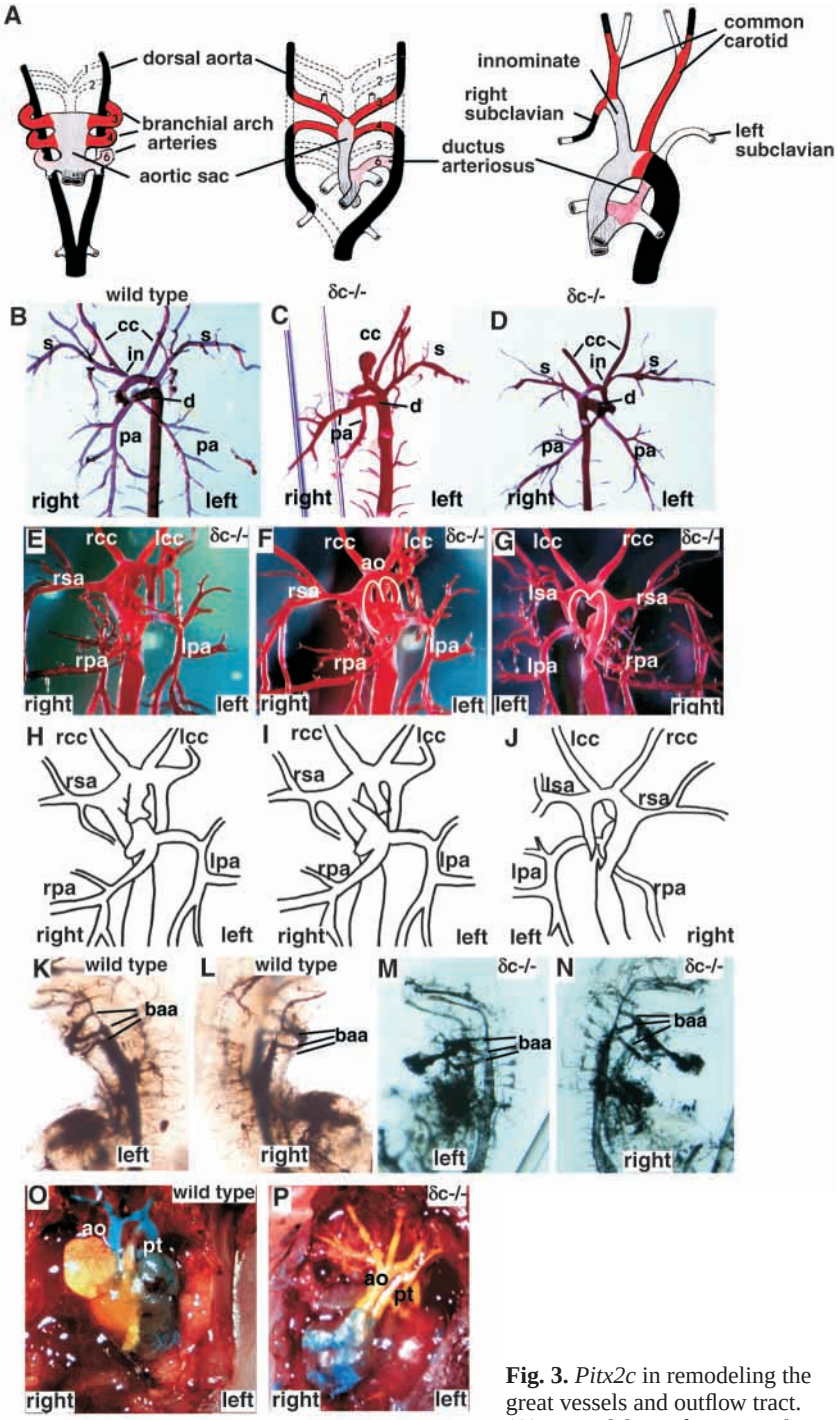

Fig. 3. *Pitx2c* in remodeling the great vessels and outflow tract. (A) Remodeling of aortic arch arteries and derivation of mature aortic arch vessels (adapted from Moore, 1982). (B-D) Corrosion cast of wild-type (B) and *Pitx2* $\delta c^{-/-}$ embryos with correct direction of aortic arch (C) and reversed orientation (D). (E-G) Corrosion cast of a *Pitx2* $\delta c^{-/-}$ embryo with double aortic arch showing ventral (E), ventral oblique (F) and dorsal (G) views. (H-J) Diagrams of the corrosion cast to more clearly show changes in vessel morphology. Each diagram is associated with the cast directly above. (K-N) India ink injection into wild-type and *Pitx2* $\delta c^{-/-}$ embryos at 11.5 dpc. Right and left oblique views are shown. (O,P) Casting dye injection into 18.5 dpc wild-type and *Pitx2* $\delta c^{-/-}$ embryos showing DORV in mutant (P). ao, aorta; baa, branchial arch arteries; cc, common carotid; d, ductus arteriosus; in, innominate artery; lcc, left common carotid; lpa, left pulmonary artery; lsa, left subclavian artery; pa, pulmonary artery; pt, pulmonary trunk; rcc, right common carotid artery; rpa, right pulmonary artery; rsa, right subclavian artery; s, subclavian artery.

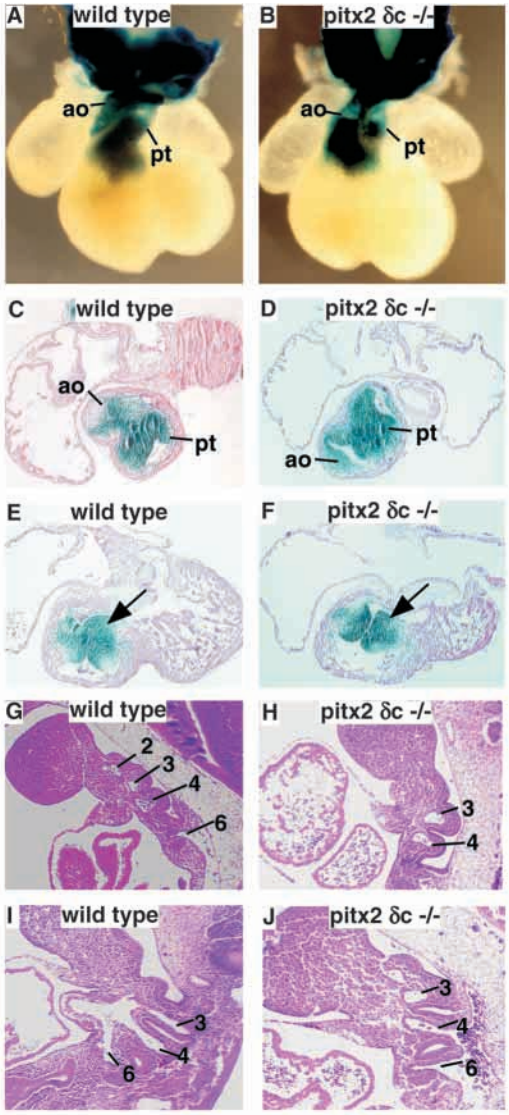

Fig. 4. Fate mapping with *Wnt1* cre transgenic and the *Rosa26* reporter. (A,B) Ventral view of whole-mount *lacZ* staining of 12.5 dpc wild-type and *Pitx2* $\delta c^{-/-}$ mutant embryo. (C-F) Rostral (C,D) and caudal (E,F) transverse section through the conotruncus of *lacZ* stained embryos showing *lacZ*-labeled cardiac crest derivatives contributing to valves and cushions of OFT (denoted by arrows). (G-J) Parsagittal sections through 10.5 dpc wild-type (G,I) and *Pitx2* $\delta c^{-/-}$ (H,J) mutant embryos at different mediolateral planes of section. Branchial arch arteries are numbered. ao, aorta; pt, pulmonary trunk.

through 16.5 dpc hearts, after *Pitx2c* expression had been extinguished, demonstrated that *lacZ*-positive *Pitx2* daughter cells populated the myocardium of the proximal OFT, as well as the remodeled membranous and muscular ventricular septum and atrial septum (Fig. 6I,J).

Analysis of the fate of *Pitx2* daughter cells in the *Pitx2* $\delta abc^{creneo};\delta c$ mutant embryos, that turned normally and survived longer than *Pitx2* null embryos, revealed that fewer *lacZ*-positive cells were found in the right ventricular and inner curvature myocardium of *Pitx2* mutant embryos at both 14.5 dpc (Fig. 6K,L) and 18.5 dpc (Fig. 6M,N). Serial transverse sections through the 18.5 dpc hearts revealed that in *Pitx2* δabc^{creneo} heterozygous hearts *lacZ*-labeled cells were found at the inferior border of the heart near the cardiac apex (Fig. 6O,Q). By contrast, in the *Pitx2* $\delta abc^{creneo};\delta c$ mutants *lacZ*-labeled cells were not found at the inferior boundary of the heart (Fig. 6P,R). Moreover, sections through 12.5 dpc hearts revealed that *lacZ*-positive cells were found in the central AV cushion of *Pitx2* δabc^{creneo} heterozygous embryos, revealing that *Pitx2* descendents contributed to the cushion mesenchyme (Fig. 6S). By contrast, in both 12.5 dpc and 14.5 dpc *Pitx2*-null mutant embryos, *lacZ*-positive cells were excluded from the central AV cushion mesenchyme suggesting that *Pitx2* function was required for invasion of *Pitx2* daughters into the AV cushion (Fig. 6T,U). As *Pitx2c* expression is never detected in endocardium, this fate mapping data suggests a myocardial source for the *lacZ*-labeled cells in the AV cushion.

Defective valve morphogenesis is a common feature in human patients with laterality defects and *Pitx2*-null embryos (Brown and Anderson, 1999; Icardo and Sanchez de Vega, 1991; Liu et al., 2001). At 16.5 dpc, *lacZ*-positive *Pitx2* descendents were detected in the AV valve leaflets of *Pitx2* δabc^{creneo} heterozygotes but were excluded from the valve leaflets of *Pitx2* $\delta abc^{creneo};\delta c$ mutants (Fig. 6V,W).

Defective pulmonary and caval vein morphogenesis in *Pitx2* mutants

Corrosion casting and scanning electron microscopy was used to analyze the morphology of the pulmonary veins in *Pitx2* mutant embryos. The left superior caval vein (LSCV) normally flows into the coronary sinus, while the right superior caval vein (RSCV) and inferior caval vein (ICV) are connected to the right atrium (RA) by thin strips at the valves. The left and right pulmonary veins join to a common pulmonary vein (PV) that drains into the left atrium (LA) (Fig. 7A,C). In most *Pitx2* $\delta c^{-/-}$ embryos, morphology of these structures was defective, with all these veins running together into a common medial venous sinus (Fig. 7B,D). Consistent with this phenotype, fate mapping with the *Pitx2* δabc^{creneo} allele showed that *lacZ*-positive *Pitx2* daughter cells were observed bilaterally in the pulmonary veins of *Pitx2* δabc^{creneo} heterozygous embryos but were severely reduced in the pulmonary veins of *Pitx2* $\delta abc^{creneo};\delta c$ mutant embryos (Fig. 7E-H).

DISCUSSION

In this work, we provide evidence that *Pitx2c* patterns a presumptive secondary heart field, the branchial arch mesoderm, that invades the heart after looping and contributes to the OFT and right ventricular myocardium. This finding is consistent with the phenotypes of the *Pitx2*-null embryos that have correct dextral looping of the heart tube but severe defects in cardiac morphogenesis (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Liu et al., 2001; Lu et al., 1999b). We also show that *Pitx2c* has an important role in asymmetric remodeling of the BAAs. Moreover, our data reveal that *Pitx2* daughter cells invade the AV cushions and valves and that this cellular movement into the cushions requires *Pitx2* function. The data presented here provide insight into the phenotypes observed in humans with laterality syndromes and demonstrate

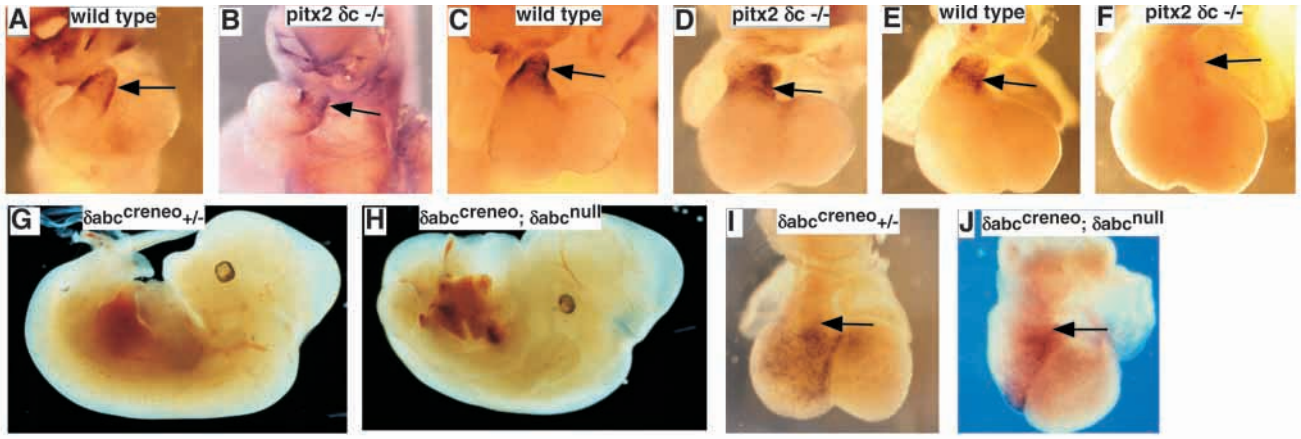

Fig. 5. Whole-mount in situ with markers of outflow tract myocardium. (A,B) 10.5 dpc wild-type (A) and *Pitx2* $\delta c^{-/-}$ (B) embryos hybridized with semaphorin 3c probe. (C,D) 11.5 dpc wild-type (C) and *Pitx2* $\delta c^{-/-}$ (D) embryos hybridized with semaphorin 3c probe showing expression in outflow tract myocardium (arrows). (E,F) 12.5 dpc wild-type (E) and *Pitx2* $\delta c^{-/-}$ (F) embryos hybridized with semaphorin 3c probe showing that expression of semaphorin 3c is reduced in the mutant (n=3) (arrows). (G,H) Whole-mount views of 12.5 dpc wild-type (G) and *Pitx2* $\delta abc^{creneo};\delta abc^{null}$ (H) null mutant embryos. *Pitx2* $\delta abc^{creneo};\delta abc^{null}$ embryos demonstrate embryonic rotation, anterior body wall closure defects and eye anomalies typical of *Pitx2* null embryos. (I,J) 12.5 dpc wild-type (I) and *Pitx2* $\delta abc^{creneo};\delta abc^{null}$ (J) embryos hybridized with *cre* probe showing expression in outflow tract and right ventricular myocardium (arrows).

a direct causal link between the genetic pathways regulating left right asymmetry and complex cardiac morphogenesis.

Pitx2 functions in the presumptive secondary heart field derived from branchial arch and splanchnic mesoderm

Recent advances have revealed that the primary heart field receives contributions from a number of secondary fields. Functional studies have implicated the cardiac neural crest in patterning of the aortic arch vessels and conotruncus of the heart. For example, mice with mutations in components of the endothelin signaling pathway (Yanagisawa et al., 1998), forkhead genes (Iida et al., 1997; Kume et al., 2001; Winnier et al., 1999) and splotch mutant mice (Epstein et al., 2000) have defective arterioventricular connections secondary to cardiac neural crest abnormalities. Moreover, inactivation of *Sema3c* and neuropilin 1 leads to faulty conotruncal cushion formation as a result of aberrant cardiac neural crest migration (Brown et al., 2001; Feiner et al., 2001; Kawasaki et al., 1999). By contrast, our data reveal that *Pitx2c* has an important role in patterning a separate heart field derived from the branchial arch and splanchnic mesoderm.

We found that *Pitx2c* is expressed asymmetrically in the left branchial arch and splanchnic mesoderm within cells that will contribute to the OFT myocardium. Moreover, *Pitx2c* is also expressed in OFT myocardium and right ventricular myocardium, regions of the heart that are populated by cells derived from branchial arch and splanchnic mesoderm, but not by cardiac neural crest (Jiang et al., 2000; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Our fate-mapping studies with the *Wnt1 cre* and *Rosa26* reporter mice also show that cardiac neural crest migrates normally in *Pitx2* mutants.

Our data suggest that *Pitx2c* is not required for the initial migration of branchial arch mesoderm into the outflow tract. Analysis of *Sema3c* expression in *Pitx2c* mutants suggests that the defect in *Pitx2c* mutants occurs relatively late in conotruncal development. Moreover, *cre* expression in *Pitx2* $\delta abc^{creneo};\delta abc^{null}$ mutants was similar to that observed in the δabc^{creneo} heterozygous OFT. One idea to explain these data is that *Pitx2* functions to maintain signaling between the outflow tract myocardium and underlying endothelium and forming conotruncal cushions. This model would be similar to what has been proposed for *Pitx2* in craniofacial development where *Pitx2* has a role in epithelial-mesenchymal signaling important for tooth organogenesis (Lin et al., 1999; Lu et al., 1999b). Another possibility, based on the ventricular myocardial defect observed in *Pitx2* mutant embryos (see below), is that *Pitx2* regulates local expansion of OFT myocardium. We favor the first hypothesis as we do not detect differences in the number or localization of *lacZ*-labeled cells in the OFT of wild-type and *Pitx2* mutant embryos. Nonetheless, it is still formally possible that subtle differences in OFT myocardial expansion could be responsible for the conotruncal defect observed in *Pitx2* mutants. Taken together, our findings support the idea that *Pitx2* patterns the branchial arch mesoderm and OFT myocardium to support normal development of the conotruncus.

Pitx2c and asymmetric remodeling of the branchial arch arteries

Development of the branchial arch arteries and subsequent remodeling into the mature aortic arch arteries involves a series of paracrine signaling events (Fishman and Kirby, 1998; Hanahan, 1997; Yancopoulos et al., 2000). The forming BAA endothelial tubes are located within the branchial arch mesoderm in close proximity to surface ectoderm and the endoderm-derived epithelium of the branchial pouches. Signaling from endothelium to mesenchyme is thought to be important for recruitment of supporting cells, such as smooth muscle precursors and pericytes, which are important for stabilization of the forming endothelial tubes (Hanahan, 1997).

Vascular remodeling involves local disruption of the critical interaction between endothelium and support cells with resulting regression of the endothelium. In one system,

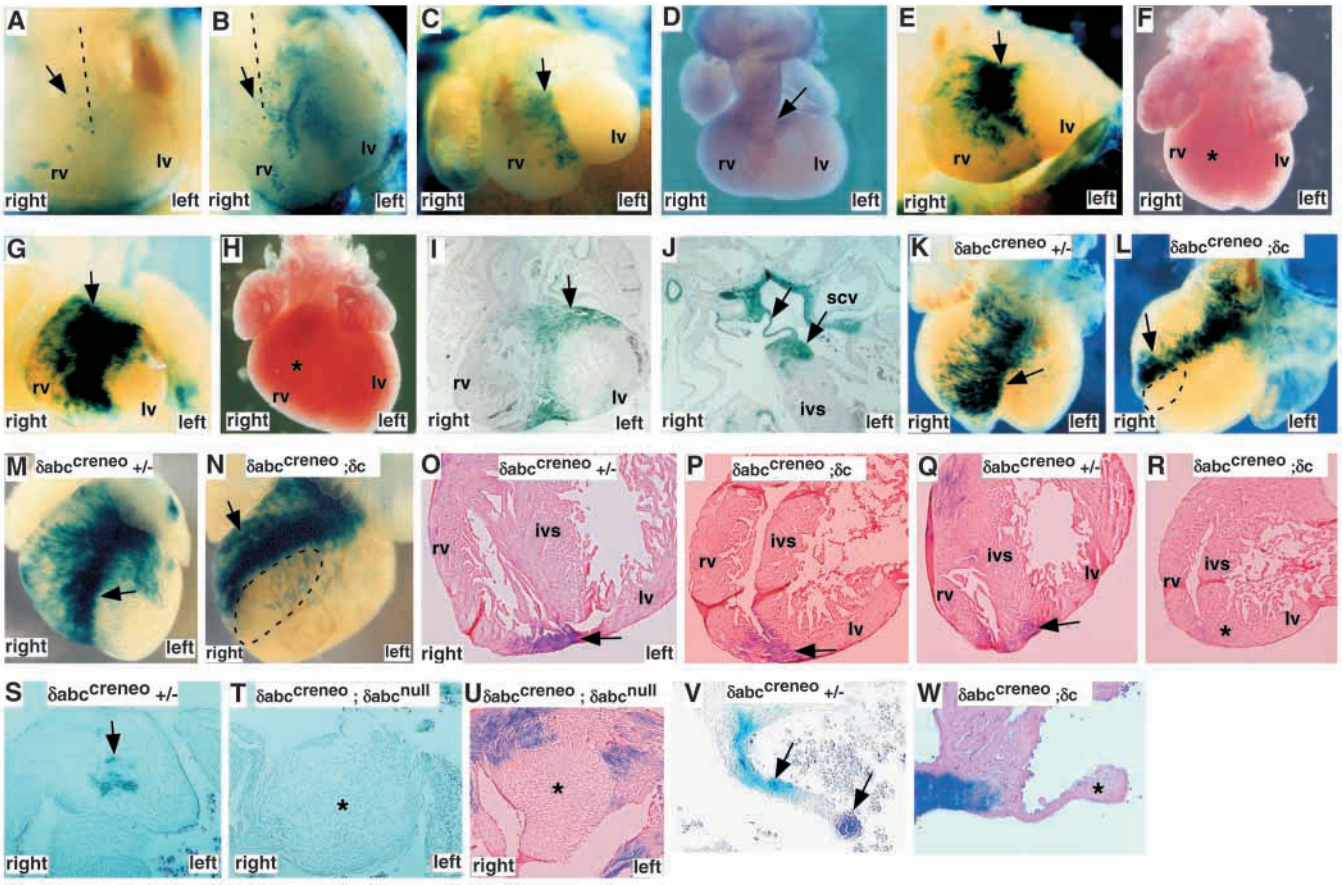

Fig. 6. Fate mapping with *Pitx2 δabc*^{creneo} and *Rosa26* reporter allele. (A,B) *lacZ* staining of 10.5 dpc *Pitx2 δabc*$^{creneo+/-}$ (A) and *Pitx2 δabc*^{creneo}*;δabc*^{null} (B), and *Rosa26* reporter *trans*-heterozygous embryo. Arrow indicates the *lacZ*-positive cells in the OFT that have crossed the midline (broken line). (C,D) *lacZ* staining (C) and *Pitx2c* whole-mount in situ (D) of 12.5 dpc *Pitx2 δabc*$^{creneo+/-}$ and *Rosa26* reporter *trans* heterozygous embryos. Signal is indicated by the arrows. (E-H) *lacZ* staining (E,G) and *Pitx2c* whole-mount in situ (F,H) of 14.5 dpc (E,F) and 16.5 dpc (G,H) *Pitx2 δabc*$^{creneo+/-}$ and *Rosa26* reporter *trans* heterozygotes. *lacZ*-positive cells are indicated by arrows (E,G). *Pitx2c* expression has been extinguished in *Pitx2* daughter cells that would be *lacZ* positive and are marked with and asterisk (F,H). (I,J) Coronal sections through a 16.0 dpc *Pitx2 δabc*$^{creneo+/-}$ and *Rosa26* reporter *trans*-heterozygotes at slightly different dorsoventral planes. Arrows indicate *lacZ*-positive cells in myocardium (I) and in interatrial and interventricular septum (J). (K,L) Whole-mount *lacZ* staining of 14.5 dpc wild-type (K) and *Pitx2 δabc*^{creneo}*;δc Pitx2* mutant (L), and *Rosa26* reporter trans-heterozygous embryos. Arrows indicate *lacZ*-positive cells. Circled area in L indicates region with fewer *lacZ*-positive cells. (M,N)Whole-mount *lacZ* staining of 16.5 dpc wild-type (M) and *Pitx2 δabc*^{crenoe}*;δc Pitx2* mutant (N), and *Rosa26* reporter *trans*-heterozygotes. Arrows indicate *lacZ*-positive cells. Circled area in N indicates region with fewer *lacZ*-positive cells. (O-R) Transverse sections through a 16.5 dpc *Pitx2 δabc*$^{creneo+/-}$ (O,Q) and *Pitx2 δabc*^{creneo}*;δc* (P,R) and *Rosa26* reporter trans-heterozygous embryo. More rostral sections show *lacZ*-positive *Pitx2* daughter cells (arrow) in myocardium of both *Pitx2 δabc*$^{creneo+/-}$ (O) and *δabc*^{creneo}*;δc* mutants (P), while more caudal sections near cardiac apex show *lacZ*-positive cells in *δabc*$^{creneo+/-}$ (Q) but not in *δabc*^{creneo}*;δc* mutant (R) as denoted by the asterisk. (S,T) Transverse sections through a 12.5 dpc *Pitx2 δabc*$^{creneo+/-}$ (S) and *Pitx2 δabc*^{creneo}*;δabc*^{null} (T) and *Rosa26* reporter *trans*-heterozygote showing *lacZ* expression in AV cushion in heterozygote (arrow) but absent in the mutant (asterisk). (U) Coronal section through a 14.5 dpc *Pitx2 δabc*^{creneo}*;δabc*^{null} and *Rosa26* reporter *trans*-heterozygote showing exclusion of *lacZ*-positive cells from AV cushion (asterisk). (V,W) Coronal section through a 16.5 dpc *Pitx2 δabc*$^{creneo+/-}$ (V) and *δabc*^{creneo}*;δc* (W) and *Rosa26* reporter *trans*-heterozygote showing *lacZ*-positive cells within valve leaflet in the *Pitx2 δabc*$^{creneo+/-}$ embryo (arrows) but exclusion of *lacZ*-positive cells from the AV valve leaflets of *Pitx2* mutants(asterisk). rv, right ventricle; lv, left ventricle; ivs, interventricular septum; scv, superior caval vein.

endothelial regression occurs by programmed cell death secondary to loss of survival factors (Meeson et al., 1996; Meeson et al., 1999). The mechanisms underlying asymmetric remodeling of the BAAs, resulting in left-sided aortic arch, are poorly understood.

Cell ablation studies in chick embryos and loss-of-function experiments performed in mice have defined a role for cardiac crest in maintaining the integrity of branchial arch arteries (Brown et al., 2001). However, recent fate mapping experiments using *Wnt1 cre* and *Rosa26* reporter mice, while confirming the importance of the cardiac crest in mouse BAA formation, suggest that cardiac neural crest does not provide the signal for asymmetric remodeling of the BAAs (Jiang et al., 2000).

The important role of branchial arch endoderm in BAA development has been illustrated by phenotypes of individuals with DiGeorge syndrome and mouse models of this syndrome that include severe defects in aortic arch artery formation

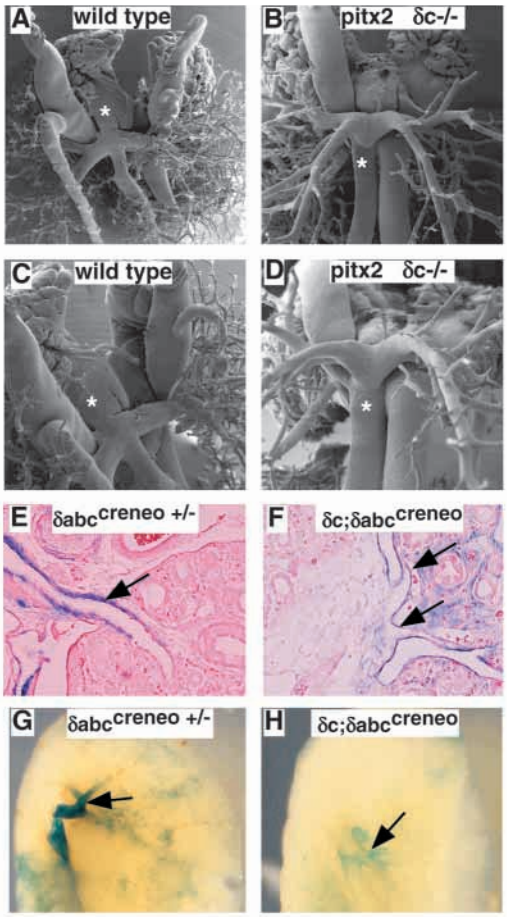

Fig. 7. Analysis of pulmonary vein morphology in *Pitx2* $\delta c^{-/-}$ embryos and fate mapping with *Pitx2* δabc^{creneo} allele. (A-D) Scanning electron microscopy of corrosion casts of wild type (A,C) and the *Pitx2* $\delta c^{-/-}$ (B,D) embryos. In wild-type embryos, the LSCV drains into the coronary sinus, guarded by the valve of the coronary sinus. The RSCV and ICV are connected to right atrium by thin strips at the valves. The left and right pulmonary veins join to a common pulmonary vein that drains into left atrium. White stars indicate left atrium, just superior to entry of common pulmonary vein (A,C). By contrast, in *Pitx2* $\delta c^{-/-}$ embryos, all these veins converge into a common, medial venous sinus (B,D). Stars indicate inferior caval vein, just inferior to entry of pulmonary vein and the left superior caval vein. This embryo also has bilateral inferior caval veins. (E-H) Fate mapping with *Pitx2* δabc^{creneo} allele. Transverse sections through lungs of 16.5 wild-type (E) and *Pitx2* mutant (F). *lacZ* positive cells marking *Pitx2* daughter cells are present in wild type but are severely reduced in mutant (arrows). (G,H) Whole mounts of 16.5 dpc lungs from wild type (G) and *Pitx2* mutants (H), showing *lacZ*-positive cells in pulmonary veins of wild type and reduced staining in mutant (arrows).

(Lindsay et al., 2001; Merscher et al., 2001). Importantly, defects were observed more commonly in the right fourth BAAs of a haploinsufficent mouse model for DiGeorge Syndrome (Lindsay and Baldini, 2001). The gene implicated in these events, *Tbx1*, is expressed in branchial arch endoderm, suggesting that endoderm-derived signals may have a role in asymmetric remodeling of BAA.

Pitx2c is expressed asymmetrically in a very discrete population of cells in proximity to the left aortic sac and left BAAs. Despite this restricted expression, there is a strong BAA phenotype in *Pitx2c* mutants. These observations suggest that *Pitx2c* may have a role in recruitment or maintenance of supporting cells to the left BAAs and aortic sac. In wild-type embryos, *Pitx2c* may be important for stabilization of left-sided BAAs, such as the sixth BAA, that will form the left-sided ductus arteriosus. In the absence of *Pitx2c* function, maintenance of the sixth BAA would be impaired, resulting in formation of a right-sided ductus arteriosus in some embryos. This alteration would initiate a cascade, perhaps resulting from the altered hemodynamics of the persistent right-sided sixth BAA, to alter remodeling of the other BAAs. Although these ideas will need to be verified in future experiments, our data provide new information about the role of *Pitx2* in asymmetric remodeling of the BAA.

Pitx2 in cushion and valve morphogenesis

Our data suggest that *Pitx2* has a greater role in AV cushion morphogenesis when compared with formation of the conotruncal cushions. *Pitx2*-null embryos have severe defects in the central mesenchymal mass that forms the AV cushions and valves resulting in complete AV canal (Kitamura et al., 1999; Liu et al., 2001). The conotruncal phenotype is a failure of rotation of the truncus arteriosus and conotruncal cushion dysmorphology (Kitamura et al., 1999; Liu et al., 2001). Genetic evidence from mice implicates Bmp-signaling in conotruncal cushion morphogenesis (Kim et al., 2001). Noggin overexpression experiments performed in chick embryos revealed that Bmp signaling in conotruncal cushion formation functioned through a mechanism involving regulation of cardiac neural crest migration (Allen et al., 2001). Less is known about the signaling pathways that regulate AV cushion morphogenesis, although recent experiments suggest that Bmp-signaling has a central role (Gaussin et al., 2002).

Data from zebrafish suggest that composition of matrix is of crucial importance in the initial formation of valves and implicate Wnt and Bmp signaling in these events (Walsh and Stainier, 2001). In vitro studies suggest that the action of matrix metalloproteases on cushion mesenchyme is required for migration of mesenchyme into the forming cushions (Song et al., 2000). This epithelial-mesenchymal transition that leads to cushion deposition requires Tgfβ signaling (Brown et al., 1996; Brown et al., 1999). In addition, Tgfβ2 null mice have multiple defects in valve and septal morphogenesis, implicating this signaling pathway in cushion morphogenesis (Bartram et al., 2001; Sanford et al., 1997). Our data reveal that *Pitx2* has a role in regulating cellular movement into the formed AV cushion, a late step in cushion morphogenesis. One idea to explain these data is that *Pitx2c* is required for the myocardial invasion of AV cushion mesenchyme. *Pitx2c* is expressed in the inner curvature myocardium that surrounds the AV cushion and these myocardial cells have been shown to invade the AV cushion mesenchyme (van den Hoff et al., 2001). However, another possible source of cells that invade the AV cushion is dorsal mesocardium that also expresses *Pitx2c*. Further experiments are currently under way to elucidate the exact source of invading *Pitx2* daughter cells.

Pitx2 function in the venous pole

The data presented here extend our previous understanding of *Pitx2* function in development of the venous pole of the heart.

Previous studies have demonstrated an important role for *Pitx2* in patterning of the atrial appendages and atrial septation (Kitamura et al., 1999; Liu et al., 2001). Analysis of the *Pitx2c* mutants also reveal a role for *Pitx2* in morphogenesis of the pulmonary and caval veins. Fate mapping suggests a direct role for *Pitx2* in vein morphogenesis as *Pitx2* daughters populate pulmonary and caval veins. Moreover, diminished contribution of *Pitx2* daughters to the *Pitx2* mutant pulmonary vein suggests a role for *Pitx2* in cell movement or cell sorting that may be similar to *Pitx2* function in AV cushion morphogenesis. Alternatively, *Pitx2* may function to regulate proliferation or survival of pulmonary vein and AV cushion progenitors.

Pitx2 function in expansion of ventricular myocardium

Pitx2c is expressed in the right ventricular and inner curvature myocardium (Campione et al., 2001; Schweickert et al., 2000). Our fate mapping experiment revealed that *Pitx2* daughter cells expand to extensively populate both right and left ventricular myocardium. In *Pitx2* mutant embryos, fewer *Pitx2* daughters are observed contributing to ventricular myocardium. Moreover, analysis of *cre* expression in *Pitx2* mutants, that marks the right ventricle, suggested that the size of the right ventricle was reduced in *Pitx2* mutants. One interpretation of these data is that *Pitx2* functions in growth of the right ventricular myocardium. Further experiments will be required to distinguish between defective movement of precursors into the right ventricle and failure of the right ventricular myocardium to proliferate.

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