

Left-right lineage analysis of the embryonic *Xenopus* heart reveals a novel framework linking congenital cardiac defects and laterality disease

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The significant morbidity and mortality associated with laterality disease almost always are attributed to complex congenital heart defects (CHDs), reflecting the extreme susceptibility of the developing heart to disturbances in the left-right (LR) body plan. To determine how LR positional information becomes ‘translated’ into anatomical asymmetry, left versus right side cardiomyocyte cell lineages were traced in normal and laterality defective embryos of the frog, *Xenopus laevis*. In normal embryos, myocytes in some regions of the heart were derived consistently from a unilateral lineage, whereas other regions were derived consistently from both left and right side lineages. However, in heterotaxic embryos experimentally induced by ectopic activation or attenuation of ALK4 signaling, hearts contained variable LR cell composition, not only compared with controls but also compared with hearts from other heterotaxic embryos. In most cases, LR cell lineage defects were associated with abnormal cardiac morphology and were preceded by abnormal *Pitx2c* expression in the lateral plate mesoderm. In situs inversus embryos there was a mirror image reversal in *Pitx2c* expression and LR lineage composition. Surprisingly, most of the embryos that failed to develop heterotaxy or situs inversus in response to misregulated ALK4 signaling nevertheless had altered *Pitx2c* expression, abnormal cardiomyocyte LR lineage composition and abnormal heart structure, demonstrating that cardiac laterality defects can occur even in instances of otherwise normal body situs. These results indicate that: (1) different regions of the heart contain distinct LR myocyte compositions; (2) LR cardiomyocyte lineages and *Pitx2c* expression are altered in laterality defective embryos; and (3) abnormal LR cardiac lineage composition frequently is associated with cardiac malformations. We propose that proper LR cell composition is necessary for normal morphogenesis, and that misallocated LR cell lineages may be causatively linked with CHDs that are present in heterotaxic individuals, as well as some ‘isolated’ CHDs that are found in individuals lacking overt features of laterality disease.

KEY WORDS: ActRIB, ALK4, Cardiac development, Cardiomyocyte, Congenital heart defect, Heterotaxy, Laterality, Left-right asymmetry, Situs inversus, TGFB

INTRODUCTION

The vertebrate body plan exhibits highly conserved asymmetries that are evident in both the consistent left-right (LR) arrangement of the heart and viscera, and in the molecular and anatomical LR differences that are characteristic of individual organs (reviewed by Levin, 2005). Deviations in normal LR pattern during embryogenesis result in a wide spectrum of abnormal laterality phenotypes that are broadly classified as either heterotaxy or situs inversus (reviewed by Bowers et al., 1996). Heterotaxy is a condition of discordant LR organ asymmetries, such that some organs are normal in their LR asymmetry, and others are not. The most severe type of heterotaxy is a condition called isomerism, in which normally asymmetric organs instead develop left or right symmetry. In contrast to heterotaxy, situs inversus is a condition in which all organs are LR reversed, resulting in a complete mirror-image asymmetry of the heart and viscera.

Many of the most severe and life-threatening complex congenital heart defects (CHDs) occur in individuals who are afflicted by laterality disease. Whereas the overall incidence of CHDs in the

general population is estimated to occur in 0.08% of live births (Ferencz et al., 1985), this incidence dramatically rises to 90% or higher for individuals exhibiting heterotaxic phenotypes (Nugent et al., 1994). CHDs in individuals with heterotaxy commonly include atrial septal defects, ventricular septal defects, transposition (or corrected transposition) of the great arteries, a double outlet right ventricle, anomalous venous return, a single ventricle and aortic arch anomalies (reviewed by Bowers et al., 1996; Bartram et al., 2005). CHDs commonly associated with isomerism include formation of two morphologically ‘leftish’ or ‘rightish’ atria, an absent or deficient atrial septum, anomalous venous return and loss of the coronary sinus (reviewed by Bowers et al., 1996; Bartram et al., 2005). Despite the concordance of organ systems in situs inversus, the incidence of CHDs in situs inversus individuals is nevertheless elevated (3% versus 0.08%) compared with that in individuals exhibiting normal body situs (situs solitus) (Ferencz et al., 1985; Nugent et al., 1994; Sternick et al., 2004). In addition, the risk for developing heterotaxy, and hence CHDs, is greatly increased for progeny of individuals with situs inversus (Burn, 1991; Gebbia et al., 1997).

The prevalence of CHDs that is associated with laterality disease indicates that heart development is greatly affected by the mechanisms driving LR body axis determination. This association is not surprising, given the many LR asymmetries that the heart must develop during its formation (reviewed by Ramsdell, 2005). During vertebrate cardiogenesis, cells located in the paired left and right splanchnic mesodermal heart fields (called the primary heart fields) coalesce to form a relatively straight tube-shaped structure

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containing an outer myocardium and an inner endocardium (reviewed by Brand, 2003; Eisenberg and Markwald, 2004). As the cardiac tube elongates through continued fusion of the primary heart fields and addition of cells from the secondary/anterior heart field, it initiates dextral (rightward and dorsal) looping morphogenesis that brings together regions of the heart tube that were initially non-adjacent to one another (reviewed by Manner, 2000). Concomitant with looping, additional cardiomyocytes are contributed from diverse sources such as the neural crest, transformation of intracardiac and extracardiac mesenchyme cells, and perhaps even the recruitment of circulating stem cells (reviewed by Eisenberg and Markwald, 2004). The rearrangement of the heart that results from looping morphogenesis is a necessary prelude to chamber formation, septation and differentiation of the inflow and outflow tracts – all processes that establish LR differences necessary for maintaining separation of systemic and pulmonary blood flow in the fully developed heart.

Using embryos of the frog *Xenopus laevis*, we recently identified a pivotal role for a type I TGF β serine-threonine kinase receptor, activin-like kinase receptor 4 (ALK4), in modulating LR axis determination and cardiac LR development. Misregulation of ALK4 signaling, through either left-side attenuation or right-side ectopic activation, causes LR reversals in heart and gut asymmetries, suggesting that ALK4 ordinarily functions on the left side of the *Xenopus* embryo to establish normal body situs (Chen et al., 2004). ALK4 signaling also regulates the classically defined *nodal*→*Pitx2c* pathway in the lateral plate mesoderm, indicating that ALK4 functions upstream of heart and visceral organ formation in modulating LR development (Chen et al., 2004). How cells of the heart use LR axis information, including that imparted by the ALK4 pathway, to generate anatomical asymmetries during organogenesis is not known.

Because the heart derives from multiple bilaterally paired sources of cells that are located to the left and right sides of the embryonic midline, we hypothesized that the regulated allocation of these left and right side lineages is an important aspect of normal cardiac LR development. To test this, we first determined the left versus right side lineage origins of all myocytes, regardless of heart field ancestry, that are present in the post-septated, looped heart. To then determine whether LR cardiomyocyte lineages are regulated by the LR body axis, results were compared with those obtained in embryos with various laterality defects, including abnormal *Pitx2c* expression, induced via ectopic activation or attenuation of ALK4 signaling. Our results define a comprehensive map of the LR myocyte composition of the vertebrate heart and show that LR lineage composition is abnormal in hearts of embryos with heterotaxy and situs inversus phenotypes. Defects in LR cardiomyocyte composition are almost always associated with cardiac malformations, demonstrating that allocation of cardiac LR lineages is an important target of LR axial patterning. We propose that the ability of cells to become differentiated into various cardiac structures is related to LR lineage origin and that when LR cardiomyocyte lineages are altered, different types of CHDs will occur depending on which region(s) of the heart are affected. In addition, because cardiac malformations, altered *Pitx2c* expression and abnormal LR cardiomyocyte compositions also are present in many of the embryos that fail to develop heterotaxy or situs inversus following experimental manipulation of ALK4 signaling, this suggests that some ‘isolated’ CHDs can nevertheless arise from subtle errors in LR patterning processes, even in the absence of overt body situs defects.

MATERIALS AND METHODS

Cell lineage labeling

Xenopus laevis embryos were produced by in vitro fertilization as described (Ramsdell et al., 1999) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Using cell size and pigmentation patterns to distinguish left and right blastomeres, Oregon Green-conjugated dextran (M_r 10,000, lysine-fixable) and Alexa 647-conjugated dextran (10,000 M_r) (Molecular Probes) were pressure injected in the marginal zone of single cells of stage 3 embryos as described (Ramsdell et al., 2005).

Induction of laterality defects

GpppG-capped RNA was transcribed with the mMessage mMachine kit (Ambion) using 300 ng of linearized CA-ALK4 or tALK4 cDNAs (Chang et al., 1997) as templates. RNA was pressure-injected into left or right ventrolateral vegetal cells of 16-cell stage embryos as described (Chen et al., 2004). After reaching stages 45–46, embryos were anesthetized with 0.01% benzocaine and scored for dorsoanterior phenotype using the dorsoanterior index (DAI) (Kao and Elinson, 1988) and LR phenotype using orientation of the heart, gut and gallbladder as indicators of body situs. Embryos exhibiting an abnormal DAI or other gross morphological defects were not used for lineage analysis.

Confocal imaging

Embryos were fixed in MEMFA (Sive et al., 2000) and processed for paraffin wax-embedded sectioning as described (Ramsdell et al., 2005). Prior to imaging the heart, embryos were prescreened at low (5–10 \times) magnification to ascertain that the dextrans were appropriately targeted only to one side of the embryo. Embryos not showing distinct LR hemi-labeling were discarded with a total of 34 prescreened embryos (control and experimental) used for lineage analyses.

Images of embryo sections were collected using a Leica TCS SP2 AOBs Confocal System mounted onto a Leica DM RE-7 upright microscope. Samples typically were viewed using a 20 \times Plan APO objective, and the dextran fluorophores were excited using either 488 nm (Argon laser) for Oregon Green or 633 nm (HeNe laser) for Alexa 647. Each tissue section was imaged at a depth giving maximum emission signal in both emission channels, and pseudo-colored green for Oregon Green and red for Alexa 647. Images were imported into Adobe Photoshop for adjustment of contrast and brightness.

In situ hybridization

Non-injected and experimental embryos were collected at stages 25–26 and processed for whole-mount in situ hybridization using a digoxigenin-labeled antisense *Pitx2c* RNA probe synthesized with the Maxiscript kit (Ambion) as described (Chen et al., 2004). Sibling embryos from control and experimental groups were maintained to monitor LR phenotypes. Images were acquired with a Spot RT camera and imported into Adobe Photoshop for adjustment of contrast and brightness.

RESULTS

To assess the stability of the fluorescent dextrans used to trace LR cardiomyocyte cell lineages, embryos were examined at stages 45–46 (~5 days of development), when the heart has undergone septation and looping morphogenesis. As shown in Fig. 1, the dextrans were not only detectable during these stages, but were also clearly restricted to the left and right sides of the embryonic midline, confirming fidelity of the lineage marker targeting. This approach differs from previous attempts to generate a LR fate map of the heart (Gormley and Nascone-Yoder, 2003; Stalsberg, 1969), in which only the primary heart fields were labeled. Because the heart comprises cells derived from multiple locations within the embryo (e.g. primary heart field, anterior/secondary heart field, neural crest, etc), labeling only the primary heart fields cannot give a comprehensive map. However, the strategy used in this study is sufficient to trace left versus right side origins of all cardiomyocytes, regardless of initial sources.

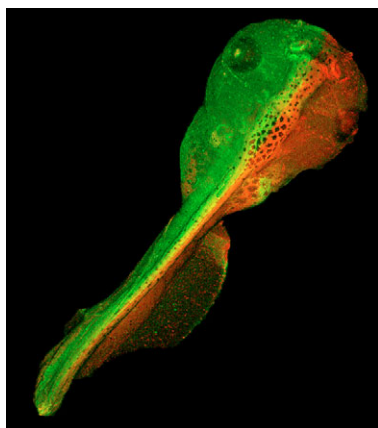


Fig. 1. Left-right lineage labeled embryo. Left and right cell lineages were microinjected with Oregon Green-conjugated dextran (green) and Alexa 647-conjugated dextran (red), respectively. Embryos were fixed and confocal images of whole mounts were collected after labeled embryos reached stages 45–46 (~5 days). A dorsal view of a labeled embryo is shown.

Images captured in serial sections showed that some regions of the heart comprised almost exclusively of either green or red cells, whereas other regions comprised both green and red cells. Beginning with the posterior (inlet) aspect of the heart, the left cardinal vein, left atrium and interatrial septum comprised green cells with red cells rarely, if ever, present in these regions, indicating that these structures were derived from the left side of the embryo (Fig. 2A). Conversely, the right cardinal vein and right atrium comprised red cells, indicating a right-side lineage composition (Fig. 2A). The myocardium of the atrioventricular canal (AVC) comprised both green and red cells; however, despite the bilateral lineages present in this region, the two lineages were segregated, with the green cardiomyocytes present in the left half of the AVC and the red cardiomyocytes present in the right half (Fig. 2B). Similar to the AVC, the ventricular myocardium comprised both green and red lineages that were segregated with respect to the midline of the heart (Fig. 2C). At the anterior (outlet) region of the heart, the muscular region of the outflow tract (OFT) and the aortic sac comprised green and red lineages that did not cross the midline of the heart (Fig. 2C); however, the right and left aortic arches comprised unilateral red and green lineages, respectively (Fig. 2C).

To determine whether cardiac LR cell lineage composition is regulated by the LR body axis, dextran-labeled embryos were microinjected with RNA encoding constitutively active ALK4 (CA-ALK4) as described (Chen et al., 2004). ALK4 is a type I serine-threonine kinase TGF β receptor that modulates LR axis determination in *Xenopus*. As previously shown (Chen et al., 2004), the majority of embryos with right-side CA-ALK4 injection had body situs defects (Table 1). Left-side expression of CA-ALK4 caused only a minority of embryos to develop body situs defects (Table 1), consistent with its role in specifying left cell lineages (Chen et al., 2004).

For all but one of the hearts obtained from situs inversus embryos, the lineage composition was a mirror-image reversal of green and red cells compared with hearts from control (non-RNA injected) embryos. Sections from a representative heart are shown in Fig. 3 in which the morphological left atrium and interatrial septum comprised red cells, indicative of a right-side origin (Fig. 3A). The morphological right atrium comprised green cells, indicating a left-side origin (Fig. 3A). The left and right cardinal veins and aortic arches comprised green and red cells, respectively. The myocardium of the AVC, ventricle, and OFT comprised green and red lineages that did not cross the midline of the heart, although owing to reversed looping (l-loop) that occurs in situs inversus embryos, these two lineages appeared mirror-imaged reversed with respect to the inner and outer curvature of the heart (Fig. 3B,C). Other than atrial inversion and reversed heart looping, no other gross morphological abnormalities were present in hearts showing complete mirror-image lineage reversals. However, one heart in this group differed in its lineage composition pattern. This exception is shown in Fig. 3, in which the morphological right atrium comprised green cells, and the morphological left atrium comprised red cells anteriorly (Fig. 3D) and green cells posteriorly (Fig. 3F,G). The two atria in this heart were separated by an interatrial septum that comprised green cells (Fig. 3D,F). Both the left and right cardinal veins comprised green cells (Fig. 3D), as were the left and right aortic arches (not shown). The myocardium of the AVC was a mirror-image reversal of the normal green and red lineages as was seen in other situs inversus embryos (Fig. 3E). However, the ventricle and proximal OFT (region nearest the ventricle) comprised exclusively green cells (Fig. 3F), although at the distal region, the OFT composition was a mix of red and green cells that did not cross the midline, but appeared mirror-imaged reversed owing to the reversed heart loop (Fig. 3G). In addition, the left and right atria were enlarged relative to the rest of the heart, and there was less distinction in size between these two chambers (Fig. 3D,F,G). The trabeculation in the ventricle was not

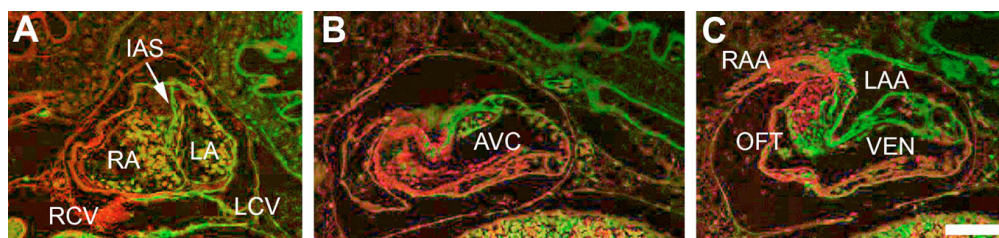


Fig. 2. Left-right lineage composition in the normal heart. Left and right cell lineages in frontal tissue sections shown in this and the following figures were labeled by Oregon Green-conjugated dextran (green) and Alexa 647-conjugated dextran (red), respectively. (A) The right atrium (RA), left atrium (LA), interatrial septum (IAS), right cardinal vein (RCV) and left cardinal vein (LCV) are shown. (B) The atrioventricular canal (AVC) is shown. (C) The right aortic arch (RAA), left aortic arch (LAA), outflow tract (OFT) and ventricle (VEN) are shown. Green- and red-labeled blood cells are in the atrial chambers (A). Scale bar: 100 μ m.

Table 1. Summary of laterality phenotypes following expression of CA-ALK4 RNA

Phenotype	Right-side RNA injection (n=26)	Left-side RNA injection (n=21)
Situs inversus	42%	5%
Heterotaxy	46%	5%
Situs solitus	12%	90%

Embryos were microinjected on the right or left-side of the embryo with CA-ALK4 RNA as described in the Materials and methods. At stages 45-46, embryos were scored for laterality phenotypes using the orientation of the heart, gut and gall bladder as indicators of body situs.

as pronounced (Fig. 3E,F; other sections not shown), and the bending of the OFT was less acute than that typically seen in either normal or other situs inversus embryos (compare Fig. 3F,G with Fig. 2C and Fig. 3C).

Unlike the relative uniformity of hearts obtained from situs inversus embryos, no two hearts obtained from heterotaxic embryos were alike with respect to morphology or LR lineage composition. LR cardiomyocyte composition and associated cardiac defects for the embryos presented in these subgroups are summarized in Table 2, where they are presented as two subgroups: (1) d-looped heart ('normal') and (2) l-looped heart ('reversed'). Three different hearts from the first heterotaxy subgroup (d-looped hearts) are shown in Fig. 4. In the first heart, the left and right cardinal veins comprised green and red lineages, respectively, that connected to a small unseptated common atrium consisting of green cells on the left side and a mixture of green and red cells on the right (Fig. 4A). The myocardium of the AVC, ventricle and OFT comprised exclusively green cells (Fig. 4C); red cells were not present in any other area of the heart except the right region of the aortic sac and the right aortic arch, the latter of which comprised a mix of red and green cells when viewed in serial sections (Fig. 4C). As in normal hearts, the left aortic arch comprised green cells (Fig. 4C). In addition to the atrial defects, this heart lacked endocardial cushion tissues (Fig. 4B,C), which normally form in both the AVC and OFT regions (e.g. compare with Fig. 4I,J), and the length of the OFT was shortened (Fig. 4B) (e.g. compare with Fig. 4I). Moreover, the ventricle contained very little trabeculation (Fig. 4B).

The second heart shown for this group also contained a small, unseptated common atrium; however, it comprised mostly red cells, with a small contribution of green cells observed at the dorsal aspect (Fig. 4D). The left and right cardinal veins (Fig. 4E) had normal lineage composition, as did the left and right aortic arches (Fig. 4G). However, the AVC myocardium comprised both green and red cells that were 'mixed' with respect to the midline of the heart, as was the ventricle (Fig. 4F,G). The proximal and distal regions of the OFT comprised almost exclusively red cells, including the aortic sac region (Fig. 4F,G). Morphologically, the common ventricle was misshapen (i.e. lacked a definitive apex) and small relative to the rest of the heart, and the OFT was shortened compared with normal (Fig. 4F,G). In addition, the ventricular trabeculation was disorganized and cushion tissues were not present in either the AVC or OFT regions (Fig. 4F,G). The third heart from this subgroup contained an unseptated common atrium that comprised green cells in the left region and red cells in the right region (Fig. 4H). The region of the common atrium containing the green cells was larger than that containing the red cells, suggesting an inversion of the rudimentary atrial chamber components. Other than this defect, the anatomy of this heart appeared normal (Fig. 4I-K), and the LR lineage composition of the remaining regions was indistinguishable from normal hearts, with the exception of some distinct patches of green cells present in the right half of the OFT (Fig. 4I).

From the second group of embryos with heterotaxy (l-looped hearts), three hearts are shown in Fig. 5. The first heart contained an unseptated common atrium that comprised green cells in its left region and red cells in the right region (Fig. 5A). No difference in size between the left and right halves was evident. The left and right cardinal veins (Fig. 5B) and left and right aortic arches (not shown) showed normal lineage derivations. The myocardium of the AVC and ventricle comprised green and red halves; these two lineages appeared inverted because of the reversed loop of the heart (Fig. 5C,D). The OFT also comprised green and red halves (Fig. 5C,D). The second heart in this subgroup contained an unseptated common atrium that comprised green and red cell regions (not shown). The remaining regions appeared anatomically normal, except for the orientation of the heart loop, and no LR lineage anomalies were observed (Fig. 5E-I). By contrast, the third heart in this subgroup contained distinct left and right atria, separated by an interatrial

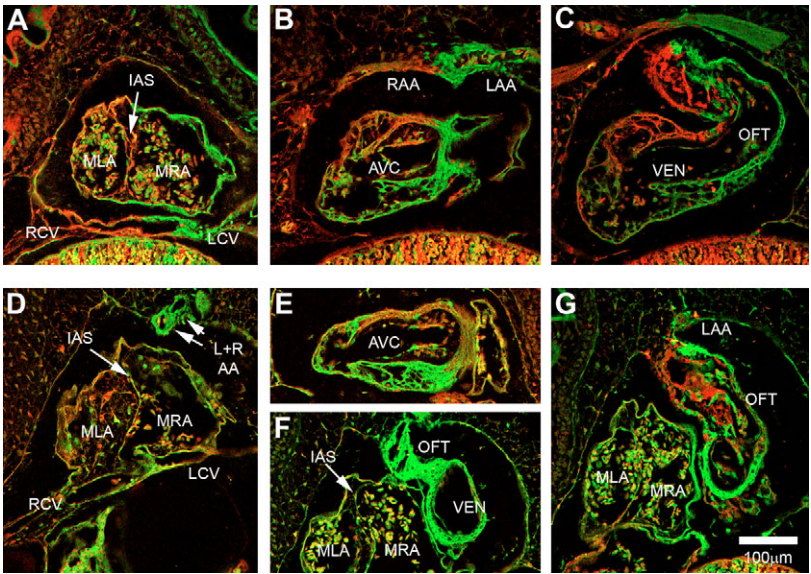


Fig. 3. Left-right lineage composition in hearts of situs inversus embryos. Two different hearts (A-C, D-G) from situs inversus embryos showing the morphological left atrium (MLA), morphological right atrium (MRA), interatrial septum (IAS), right cardinal vein (RCV), left cardinal vein (LCV), right aortic arch (RAA), left aortic arch (LAA), atrioventricular canal (AVC), ventricle (VEN) and outflow tract (OFT). The anatomical positions of the left and right atria plus looping orientation are inverted in these hearts, and labeled blood cells are in the atrial chambers (A,D,G). Scale bar: 100 μ m.

Table 2. Summary of cell lineage analyses and associated morphological defects in hearts from normal embryos and embryos with *CA-ALK4* expression

Phenotype	Heart loop	LCV	RCV	LA	RA	IAS	AVC	VEN	OFT	AS	LAA	RAA
Situs solitus ^{††}	D-loop	G	R	G	R	G	G+R	G+R	G+R	G+R	G	R
Situs inversus	L-loop	G	R	R**	G**	R	G+R	G+R	G+R	G+R	G	R
Situs inversus	L-loop	G	G	R (anterior) G (posterior)**	G**	G	G+R	G[§]	G (proximal) G+R (distal)[†]	G+R	G	G
Heterotaxy	D-loop	G	R	G	G+R	Absent	G*	G[§]	G*[†]	G+R	G	G+R
Heterotaxy	D-loop	G	R	Mostly R	Mostly R	Absent	G+R*.††	G+R[§]††	Mostly R*	Mostly R	ND	ND
Heterotaxy	D-loop	G	R	R**	G**	Absent	G+R	G+R	G+R	G+R	G	R
Heterotaxy	L-loop	G	R	G^{††}	R^{††}	Absent	G+R	G+R	G+R	G+R	G	R
Heterotaxy	L-loop	G	R	G^{††}	R^{††}	Absent	G+R	G+R	G+R	G+R	G	R
Heterotaxy	L-loop	G	R	G**	G**	G	G+R	G+R	G (proximal) R+G (distal)*.†.‡	G+R	G	R
Situs solitus ^{§§}	D-loop	G	R	G	R	G	G+R	G+R	G+R	G+R	G	R
Situs solitus ^{§§}	D-loop	G	R	R**	G**	Absent	G+R	G+R	G+R	G+R	G	R
Situs solitus ^{§§}	D-loop	G	R	R**	G**	R	G+R	G+R	G+R	G+R	G	R
Situs solitus ^{†††}	D-loop	G	R	G	G	Absent	Mostly G*	Mostly G Except G+R present in trabeculae	G (proximal) G+R (distal)[†]	G+R	G	R
Situs solitus ^{†††}	D-loop	G	R	G	G+R	G	G+R	G (left half) G+R (right half)	G+R	G+R	G	R
Situs solitus ^{†††}	D-loop	ND	ND	G**	G+R**	Absent	G+R	G (left half) G+R (right half)	G+R	G+R	G	R

Bold entries indicate abnormalities in LR lineage composition and/or morphology. G and R indicate left-side and right-side cardiomyocyte lineages, respectively. Empty boxes indicate structures for which lineage and/or morphology could not be clearly determined.

*Cushion tissue absent or reduced.

†Shortened OFT.

‡Abnormal OFT looping.

§Abnormal trabeculation.

††Abnormal size or shape.

**Inverted anatomical position.

†††Cells mixed across the cardiac midline.

‡‡Non-RNA injected.

§§Right-side *CA-ALK4* expression.

†††Left-side *CA-ALK4* expression.

ND, not determined.

septum; however, the anatomical positions of the atria were inverted and both atrial chambers and the interatrial septum comprised exclusively green cells (Fig. 5J). The cardinal veins (Fig. 5J) and aortic arches (Fig. 5L) in this heart had normal unilateral lineage compositions, and the myocardium of the AVC and the ventricle were normal in morphology and lineage composition (Fig. 5K and other sections not shown). However, the OFT region was shortened and incompletely looped; the proximal OFT comprised nearly exclusively green cells, with red cardiomyocytes present only in the distal-most region just adjacent to and including the aortic sac (Fig. 5L and other sections not shown). Endocardial cushion tissue formation appeared reduced in the OFT region (Fig. 5L) but not in the AVC.

We next examined hearts from the minority of embryos that exhibited normal body situs (situs solitus) following right-side *CA-ALK4* expression. Surprisingly, most embryos in this group exhibited some combination of morphological and cell lineage defects (summarized in Table 2), with only one embryo containing a heart that was indistinguishable from hearts of control embryos (Fig. 6A-C). Also shown in Fig. 6 is a heart that contained an unseptated common atrium comprising mostly green cells, with far fewer red cells located within the smaller right ventral region (Fig. 6D). The differing sizes of the two rudimentary atrial chamber components plus the reversed lineage contributions suggest that atrial inversion had occurred. The left and right cardinal veins (Fig.

6D) and aortic arches (Fig. 6E) comprised unilateral green and red lineages, respectively, and the morphology and lineage composition of the myocardium of the AVC, trabeculated ventricle, OFT and aortic sac were normal (Fig. 6E,F). The other abnormal heart shown for this group exhibited definitive atrial inversion (Fig. 6G). The two atrial chambers also had cell lineage inversions and the interatrial septum comprised red cells, as was previously observed for most of the hearts obtained from situs inversus embryos. No other defects, either in anatomy or in cardiomyocyte cell lineages, were present (Fig. 6H,I).

Unlike ectopic right-side *CA-ALK4* expression, left-side *CA-ALK4* expression does not cause body situs defects because this is the side of the embryo on which *ALK4* ordinarily functions (Chen et al., 2004). As expected, the majority of embryos exhibited normal body situs following left-side *CA-ALK4* expression (Table 1). However, close examination of hearts from situs solitus embryos in this group revealed that almost all contained some combination of morphological and cell lineage abnormalities (Table 2). One heart in this group contained an unseptated common atrium that comprised exclusively green cells (Fig. 7A). The AVC and ventricle comprised nearly all green cells except for some red cells mingled with green cells in the ventricular trabeculae (Fig. 7B,C). The OFT predominantly comprised green cells in the proximal region (not shown), which appeared shortened relative to the proximal OFT in normal hearts (Fig. 7B,C). In the distal OFT and the aortic sac,

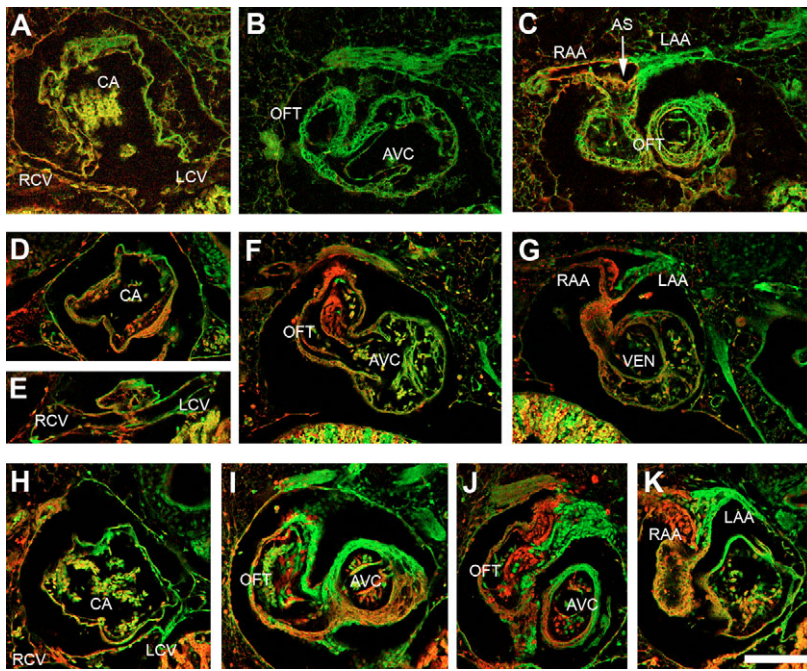


Fig. 4. Left-right lineage composition in d-looped hearts of heterotaxic embryos. Three different hearts (A-C, D-G, H-K) show the right cardinal vein (RCV), left cardinal vein (LCV), right aortic arch (RAA), left aortic arch (LAA), atrioventricular canal (AVC), ventricle (VEN) and outflow tract (OFT). All of the hearts in this category contained an unseptated common atrium (CA). Scale bar: 100 μ m.

green and red cells, which did not mix across the midline of the heart, were present (Fig. 7B,C). The OFT endocardial cushions were formed; however, endocardial cushion tissue formation was detected only in the left-derived region of the AVC region (i.e. only the 'inferior' cushion was present) (Fig. 7B). The left and right cardinal veins and aortic arches had normal unilateral lineage compositions. Another heart in this group contained two atria of similar size separated by an interatrial septum; the atrial chambers and the interatrial septum comprised green cells, with only a few red cells present in the right-side wall of the right-side atrial chamber (Fig. 7D). The left and right cardinal veins (not shown) and aortic arches (Fig. 7F) appeared normal in this heart. The lineage composition of the AVC myocardium was normal and cushion tissues were present in this region (Fig. 7E-G). However, despite normal morphological appearance of the ventricular myocardium, it comprised primarily green cells in its left half and

a mix of mostly green cells with some red cells in its right half (Fig. 7E-G). The OFT and the aortic sac, like the AVC, comprised green and red cells that did not cross the midline of the heart, and endocardial cushion tissue was present (Fig. 7E-G). Yet another heart from this group contained an unseptated common atrium and comprised green cells in its left region and a mixture of green and red cells in its right side region (Fig. 7H). The region of the common atrium containing the green cells was larger than that containing the red cells, suggesting an inversion of the rudimentary atrial chamber components. A normal contribution of green and red cells to each half of the AVC and OFT myocardium was present (Fig. 7I,J), but the ventricular myocardium comprised exclusively green cells in its left half with a mixture of mostly green cells with some red cells in its right half (Fig. 7I). Despite this abnormality in ventricular lineage composition, no gross morphological defects could be discerned in this region. Yet another heart in this group

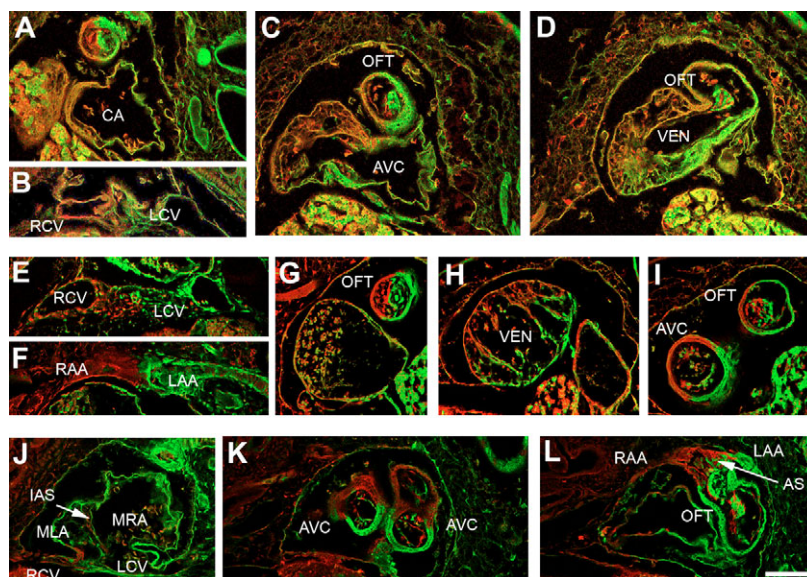


Fig. 5. Left-right lineage composition in l-looped hearts of heterotaxic embryos. Three different hearts (A-D, E-I, J-L) show the right cardinal vein (RCV), left cardinal vein (LCV), right aortic arch (RAA), left aortic arch (LAA), atrioventricular canal (AVC), ventricle (VEN) and outflow tract (OFT). The heart in A-D contains a common atrium (CA) and the heart in J-L has inverted atria, as indicated by the position of the morphological left atrium (MLA) and morphological right atrium (MRA), which are separated by an interatrial septum (IAS). Scale bar: 100 μ m.

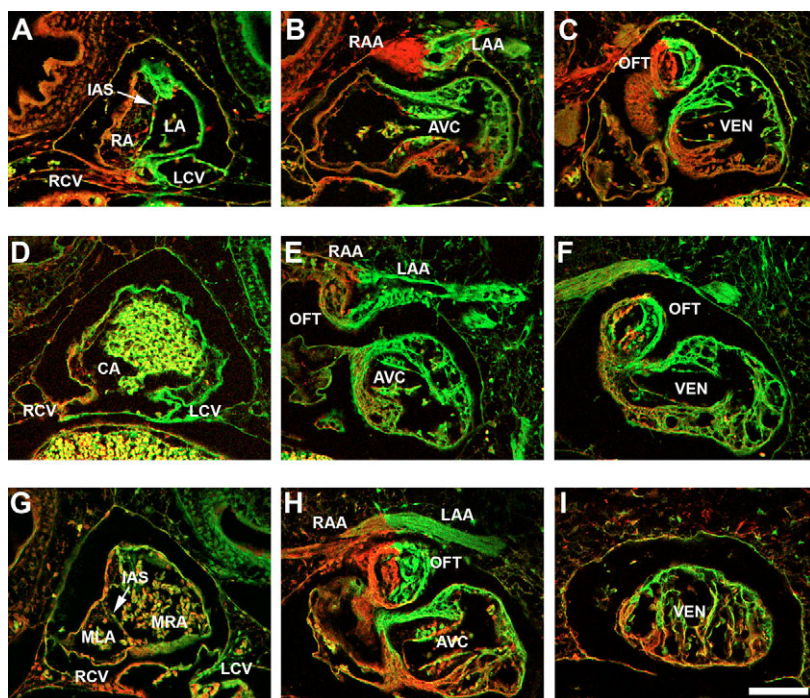


Fig. 6. Left-right lineage composition in hearts of experimental situs solitus embryos with right-side *CA-ALK4* expression. Three different hearts (A-C, D-F, G-I) are shown. The right cardinal vein (RCV), left cardinal vein (LCV), right aortic arch (RAA), left aortic arch (LAA), atrioventricular canal (AVC), ventricle (VEN), and outflow tract (OFT) are indicated. The heart in A-C contains a normally developed left atrium (LA) and right atrium (RA) separated by an interatrial septum (IAS). The heart in D-F contains a common atrium (CA) and the heart in G-I has inverted atria, as indicated by the position of the morphological left atrium (MLA) and morphological right atrium (MRA), which are separated by an IAS. Scale bar: 100 μ m.

differed from all others in that it appeared normal in every respect, including cell lineage compositions that were identical to those observed in control hearts (not shown).

Because cardiac LR lineage contributions were abnormal in most experimental situs solitus embryos, this suggested that *CA-ALK4* expression nevertheless had subtly perturbed LR axis determination. To test this, *CA-ALK4*-injected embryos were examined by whole-mount in situ hybridization for expression of *Pitx2c*. *Pitx2c* is a downstream target gene in the LR pathway that is ordinarily expressed in the left, but not right, lateral plate mesoderm (reviewed by Levin, 2005; Ramsdell, 2005) ($n=10$; Fig. 8A). Consistent with previous work (Chen et al., 2004) and with the mix of heterotaxy and situs inversus phenotypes caused by right-side *CA-ALK4* targeting,

the location of *Pitx2c* expression was bilateral (38%) or right-side only (54%) ($n=13$; Fig. 8B,C). In addition, the intensity and area of staining also were elevated compared with controls. By contrast, the majority of embryos (85%) with left-side *CA-ALK4* targeting had *Pitx2c* expression exclusively on the left side of the embryo ($n=13$; Fig. 8D). However, as observed for the right-side *CA-ALK4*-injected embryos, the intensity and extent of staining were markedly increased, with a dorsal and/or posterior expansion of staining (Fig. 8D). This subtle alteration in the amount and extent of *Pitx2c* expression in *CA-ALK4*-injected embryos was not appreciated in our previous study and suggests that the duration and/or dose, in addition to asymmetry, of ALK4 signaling are important for normal LR development.

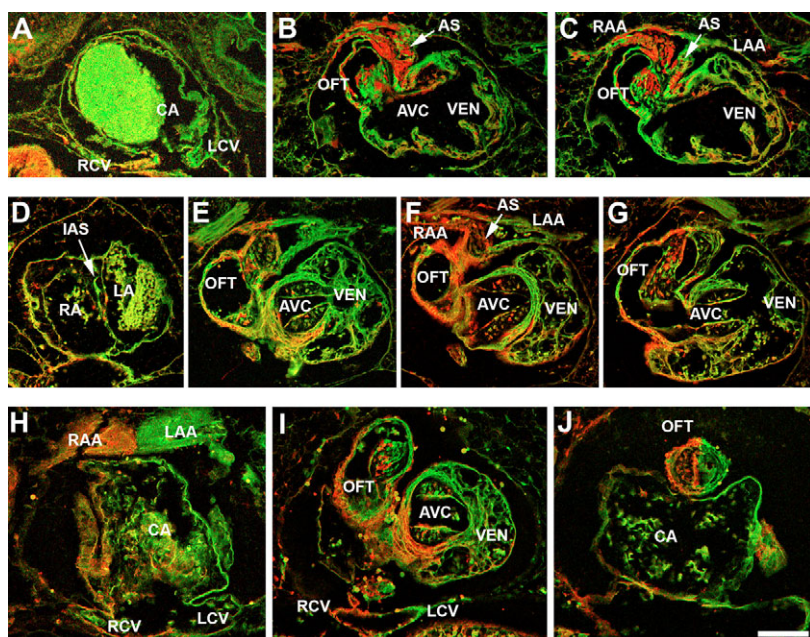


Fig. 7. Left-right lineage composition in hearts of experimental situs solitus embryos with left-side *CA-ALK4* expression. Three different hearts (A-C, D-G, H-J) are shown. The right cardinal vein (RCV), left cardinal vein (LCV), right aortic arch (RAA), left aortic arch (LAA), atrioventricular canal (AVC), ventricle (VEN), outflow tract (OFT) and aortic sac (AS) are indicated. The hearts in A-C and H-J each contain an unseptated common atrium (CA). Scale bar: 100 μ m.

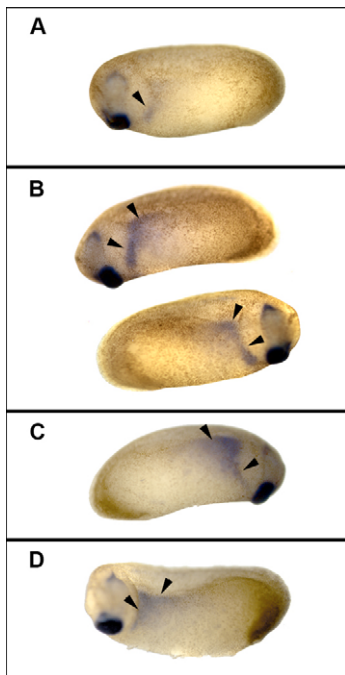


Fig. 8. *Pitx2c* expression in normal and laterality defective embryos. Control and *CA-ALK4*-injected embryos were processed for whole-mount in situ hybridization to detect *Pitx2c* expression (arrowheads). (A) Control embryo, left-side view. (B) Right-side injected embryo, left and right-side views. (C) Right-side injected embryo, right-side view. (D) Left-side injected embryo, left-side view.

To test this directly, lineage analyses were performed in embryos injected with RNA encoding a kinase-deficient version of *ALK4* that functions as a dominant-negative (DN) receptor. When expressed on the left side of the embryo, *DN-ALK4* causes LR organ reversals in approximately one-third of injected embryos, with the remaining embryos exhibiting normal body situs (Chen et al., 2004). Similar to hearts from heterotaxic embryos caused by *CA-ALK4* expression, no two hearts obtained from embryos in the *DN-ALK4*-induced heterotaxy group were exactly alike. The atrium was the most frequently affected region, with abnormal lineage composition present in five out of six embryos examined. One embryo from this group is shown in which the left and right atria were inverted (Fig. 9A). The morphological left atrium and the interatrial septum comprised a mixture of red and green cells, and the morphological right atrium comprised green cells only (Fig. 9A). The left and right cardinal veins comprised correct unilateral lineages, and the AVC comprised green and red lineages that segregated with respect to the cardiac midline (Fig. 9A,B). The ventricle also comprised green and red lineages; however, the two lineages mixed across the cardiac midline and abnormal trabeculation was present (Fig. 9C). The OFT of this heart also showed mixing of green and red cells across the cardiac midline (Fig. 9C), although the two lineages remained segregated at the level of the aortic sac (not shown). No abnormalities were noted for the aortic arches. Defects in left-right myocyte lineage allocation and/or cardiac anatomy present in other heterotaxic embryos in this group are summarized in Table 3.

The final group of hearts examined in this study was obtained from the embryos that showed situs solitus following left-side *DN-ALK4* expression. The majority of these embryos contained defects in cardiac cell lineage composition and/or anatomy (summarized in

Table 3), and two hearts from this group are shown in Fig. 9. Although the left atrium and interatrial septum of the first heart had normal, green cell composition, the right atrium contained both green and red cells (Fig. 9D). The cardinal veins showed normal lineage composition, as did the aortic arches (Fig. 9D). The AVC comprised green and red cells that mixed across the cardiac midline in the right half (Fig. 9E), but the trabeculated ventricle appeared normal, with green and red lineages segregated with respect to the midline of the heart (Fig. 9F). The OFT of this heart was shortened and contained both green and red cells that were mixed in the proximal (Fig. 9E) but not distal region (not shown). Another heart in this group contained two septated atria that did not show the pronounced differences in size and shape (Fig. 9G) observed in the atria of control embryos (Fig. 2A). Whereas the anatomical left atrium in this heart comprised green cells, the anatomical right atrium contained an abnormal mix of green and red cells. The AVC myocardium contained green cells on the left half and a mix of green and red cells on the right, and the endocardial cushions were hyperplastic (Fig. 9H). The ventricular myocardium also showed mixing of cells across the midline, with the left half of the heart comprised green cells and the right side comprised green and red cells (Fig. 9I). The ventricle in this heart appeared smaller and misshapen at the apex (Fig. 9I and other sections not shown). Mostly green cells were present in the proximal OFT (Fig. 9I), but segregated green and red cells were in the left and right regions of the very distal OFT and aortic sac (not shown). Thus, as with the situs solitus embryos in the *CA-ALK4*-injected group, the majority of embryos showing situs solitus after left-side *DN-ALK4* expression also had varying types of cell lineage and morphological defects.

DISCUSSION

Although significant progress has been made in defining mechanisms of vertebrate LR axis determination, much less is known about how organ primordia use positional information set forth by the LR axis to generate anatomical asymmetries (reviewed by Levin, 2005; Ramsdell, 2005). Our results demonstrate that allocation of LR cell lineages to the heart is an important target of LR axial patterning. When LR axis determination is compromised, as occurred in the heterotaxic and situs inversus embryos generated in this study, LR cell lineage composition of the heart is always abnormal. Moreover, even subtle perturbations of the LR developmental pathway can cause LR cell lineage abnormalities, as occurred in many of the experimental embryos that exhibited situs solitus following activated or attenuated *ALK4* signaling. Close examination of the cardiac anatomy of the laterality defective and experimental situs solitus embryos showed that cardiac LR lineage defects almost always are associated with the presence of CHDs in the affected region(s), suggesting that proper allocation of LR cell lineages is an important step in normal heart morphogenesis.

Despite the symmetric appearance of the AVC, ventricle, OFT and the paired cardinal veins and aortic arches, these regions exhibit distinct LR differences in cell lineage compositions. Regions of the heart that are morphologically asymmetric, such as the left and right atria, also exhibit differences in LR lineage compositions. Thus, with respect to the anteroposterior axis of the heart, each region exhibits a particular cellular laterality, with some regions comprised unilateral lineages and others comprised dual lineages (summarized in Fig. 10).

To determine whether allocation of cardiac cell lineages is regulated by the LR body axis, LR lineage composition was compared between hearts from control embryos and hearts from laterality defective embryos. Hearts from all laterality defective

Table 3. Summary of cell lineage analyses and associated morphological defects in hearts from embryos with *DN-ALK4* expression

Phenotype	Heart loop	LCV	RCV	LA	RA	IAS	AVC	VEN	OFT	AS	LAA	RAA
Heterotaxy	L-loop	G	R	G	G+R[¶]	G+R	G+R^{††}	G+R^{§,††}	G+R^{††}	G+R	G	R
Heterotaxy	L-loop	G	R	G	G+R	Absent	G+R	G+R^{††}	G+R	G+R	G	R
Heterotaxy	L-loop	G	R	G+R^{**}	G^{**}	Absent	G+R^{*,††}	G+R^{§,††}	G+R^{*,††}	G+R^{††}	G	R
Heterotaxy	L-loop	G	R	G+R^{**}	G^{**}	G+R	G+R	G+R^{§,††}	G+R^{††}	G+R	G	R
Heterotaxy	L-loop	G	R	G	G+R^{¶,***}	R	G+R	G+R	G+R	G+R	G	R
Heterotaxy	L-loop	G	R	G	R	G+R	G+R	Mostly G[§]	Mostly G^{†,‡}	G+R	G	R
Situs solitus	D-loop	G	R	G	Mostly R	G	G+R	G	G+R^{††}	G+R	G	R
Situs solitus	D-loop	G	R	G	R	G	G+R	G+R	G+R	G+R	G	R
Situs solitus	D-loop	G	R	G	R	G	G+R	G+R	G+R	G+R	G	R
Situs solitus	D-loop	G	R	G	Mostly G[¶]	G	G+R	G+R	G+R	G+R	G	R
Situs solitus	D-loop	G	Mostly R	G	Mostly R[¶]	G	G+R^{††}	Mostly G	G+R^{††}	G+R	G	R
Situs solitus	D-loop	G	R	G	G+R[¶]	G	G+R^{††}	G+R	G+R (proximal)^{†,††} G+R (distal) G+R^{††}	G+R	G	R
Situs solitus	D-loop	G	R	Mostly G[¶]	G[¶]	G	G+R^{*,††}	G+R^{¶,††}	G+R^{††}	G+R	G	R

Bold entries indicate abnormalities in LR lineage composition and/or morphology. G and R indicate left-side and right-side cardiomyocyte lineages, respectively.

*Cushion tissue reduced or increased.

†Shortened outflow tract.

‡Abnormal outflow tract looping.

§Abnormal trabeculation.

¶Abnormal size or shape.

**Inverted anatomical position.

††Cells mixed across the cardiac midline.

embryos induced by misregulated ALK4 signaling contained LR cell lineage defects that were preceded by altered *Pitx2c* expression in the lateral plate mesoderm. *Pitx2c* is a well-characterized laterality gene that is a crucial downstream target of symmetry-breaking events during vertebrate LR axis determination (reviewed by Levin, 2005; Ramsdell, 2005). *Pitx2c* ordinarily is expressed in the left, but not right, lateral plate mesoderm just prior to cardiogenesis. The asymmetric expression pattern of *Pitx2c* was inverted in situs inversus embryos, and in all but one situs inversus embryo, hearts had completely inverted LR lineage composition that corresponded with mirror-image reversed cardiac anatomy. In heterotaxic embryos, *Pitx2c* expression was typically inverted or bilateral, reflecting the heterogeneity of LR phenotypes that is characteristic of this condition. With respect to morphology and cell lineage composition, no two hearts from heterotaxic embryos had exactly the same phenotype, indicating that some regions of the heart were mis-specified for cellular laterality without other regions being affected. This suggests not only that each region of the heart can become independently LR specified, but also that, in instances of heterotaxy, specification of the different cardiac regions is random, as occurs on the gross anatomical level when organs develop asymmetries that are stochastic in their LR orientation. One important implication of this finding is that the pleiotropic CHDs typically found in individuals with heterotaxy (especially those with the same genetic etiology) could be attributed to developmental errors that cause discordance in this LR specification process.

Abnormal LR cardiomyocyte compositions were frequently associated with CHDs, which suggests that the ability of different regions of the heart to become differentiated into particular structures is linked to cardiac cell lineage. For example, the left and right atria derive from a common progenitor, the common atrium, which must become divided into two chambers with distinct LR differences (Anderson, 1992; Markwald et al., 1998; Min et al.,

2000). LR labeling of the primary heart fields previously suggested that this division is related to the LR origin of cells that contribute to this region (Gormley and Nascone-Yoder, 2003). Our results not only confirm this interpretation by showing that each atrium is derived from a unilateral lineage, but also provide direct evidence that atrial chamber formation rarely proceeds normally if cell lineage composition in the common atrium is altered. The two morphological defects that most often accompanied lineage defects in this region were atrial inversion, which was most commonly seen in situs inversus hearts, and atrial chamber isomerism, which was most commonly seen in hearts from heterotaxic embryos.

Studies of the interatrial septum suggest that its formation also is related to differences in the ability of left versus right-side-derived cells to become differentiated. Myocardial cells of the interatrial septum (IAS) share common gene expression with cells in the left but not right atrial wall, suggesting that the IAS and left atrial chamber both form from a cell population specified for 'leftness' (Franco and Campione, 2003; Liu et al., 2002; Wessels et al., 2000). In support of this possibility, these two cardiac components ordinarily comprised exclusively left-derived cells and the cell lineage compositions of the morphological left atrium and interatrial septum are concordantly reversed in situs inversus hearts (i.e. they are both right-side derived). Moreover, in instances where left-side signaling is impaired (i.e. some of the heterotaxic embryos), the IAS did not form. The latter experimental result correlates with clinical observations that the IAS is oftentimes either deficient or completely absent in hearts of individuals with heterotaxy (reviewed by Bowers et al., 1996; Bartram et al., 2005).

Other aspects of cardiac morphogenesis also may be dependent upon proper allocation of LR cardiomyocyte lineages. When LR cardiomyocyte lineage abnormalities were present in the AVC, endocardial cushion tissue formation frequently was absent or

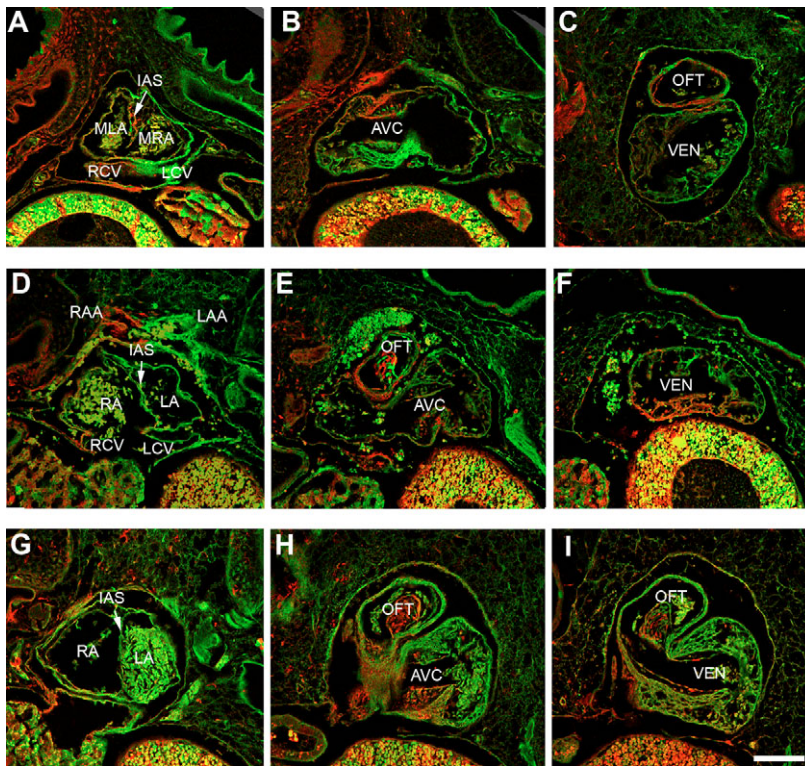


Fig. 9. Left-right lineage composition in hearts of embryos *situs* following left-side *DN-ALK4* expression. Three different hearts (**A-C**, **D-F**, **G-I**) are shown. The right cardinal vein (RCV), left cardinal vein (LCV), right aortic arch (RAA), left aortic arch (LAA), atrioventricular canal (AVC), ventricle (VEN), outflow tract (OFT) and aortic sac (AS) are indicated. Scale bar: 100 μ m.

greatly diminished. Because endocardial cushions ultimately become remodeled into valve and septal tissues, deficiencies in endocardial cushion tissue formation are a leading cause of valvuloseptal defects (reviewed by Person et al., 2005). A previous study of the endocardial cushions in the AVC of the chick embryo demonstrated that consistent with their initial left- and right-side origins, the inferior and superior cushions exhibit distinct properties throughout septation morphogenesis, including differences in proliferative rates, spatial distribution and amount of endocardial tissue formed (Moreno-Rodriguez et al., 1997). As the AVC myocardium is the primary source of signals that induce endocardial cushion tissue formation (reviewed by Person et al., 2005), it is possible that LR cardiomyocyte composition in the AVC is causatively related to the asymmetries reported for inferior versus superior cushion formation and differentiation.

In addition to the AVC, the OFT region is another site for endocardial cushion tissue formation (reviewed by Delot, 2003). As was found in hearts with defective AVC cushion formation, correlation between abnormal LR cell lineage composition and absent/reduced cushion tissue also was present in the OFT region of several hearts from heterotaxic embryos. In some (but not all) of these hearts, the cell lineage aberrancies were correlated with formation of a shortened, abnormally looped OFT. Double-outlet right ventricle, transposition of the great arteries and ventricular septal defects are types of CHDs that can result from errors in length and/or rotation of the OFT during its formation (reviewed by Hutson and Kirby, 2003). Relevant to these findings, *Pitx2* loss of function in *Xenopus* causes anomalous shifting of the OFT during looping morphogenesis (Dagle et al., 2003), suggesting that normal formation and positioning of the OFT is indeed affected by LR patterning events during vertebrate cardiogenesis.

Other regions of the heart that were affected in the laterality defective embryos included the ventricle, aortic sac and aortic arches. Disorganized and/or decreased trabeculation were present in the ventricles of some hearts that had either unilateral (left-side) lineage composition or abnormally 'mixed' left and right lineages that each were distributed on both sides of the midline of the heart. However, in other hearts, abnormal LR lineage composition was present in the ventricle, but the size and shape of the ventricle as well as ventricular trabeculation appeared grossly normal. In these hearts, as well as the few others showing lineage defects without obvious malformations, it is possible that subtle abnormalities were present but not readily detectable by histological examination (e.g. conduction or other physiological defects). Alternatively, it is possible that the cell lineage defects were not sufficiently abnormal to cause detrimental development. In addition to the ventricle, the aortic sac and the aortic arches were two other regions in which lineage defects were observed in a small minority of laterality

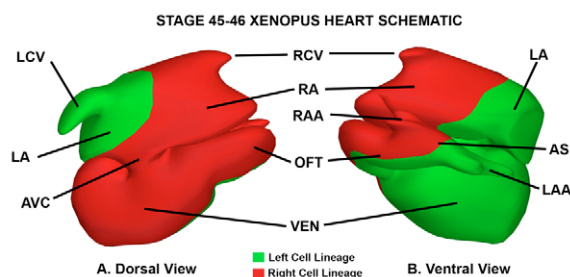


Fig. 10. LR lineage composition of the normal heart. A three-dimensional schematic heart was created based on reconstructed serial sections of several hearts in this study. The exterior was pseudo-painted green and red to indicate left and right-side cell lineages, respectively. The right cardinal vein (RCV), left cardinal vein (LCV), right aortic arch (RAA), left aortic arch (LAA), left atrium (LA), right atrium (RA), atrioventricular canal (AVC), ventricle (VEN), outflow tract (OFT) and aortic sac (AS) are indicated in dorsal (**A**) and ventral (**B**) views.

defective embryos. Again, obvious morphological defects were not present in these cases. It is possible that either the abnormalities in cell lineage composition were too minor to cause significant structural defects, or alternatively, that with continued development, CHDs reflective of dysmorphogenesis in these regions (e.g. aortic arch anomalies) might have been found. Because dextran lineage markers lose stability after approximately stage 46 due to lysosomal degradation (Sive et al., 2000), it was not possible to distinguish between these possibilities.

The other type of embryos that were examined for cardiac lineage composition were experimental embryos that failed to develop heterotaxy or situs inversus phenotypes in response to misregulated ALK4 signaling. Much to our surprise, the majority of embryos in this group exhibited LR cardiac lineage defects, despite a situs solitus phenotype. LR lineage defects were prevalent in the atrial regions, and were associated with abnormal atrial chamber development and absent interatrial septation. In addition, the ventricles of many of these embryos were altered in LR cardiomyocyte lineage composition, although obvious morphological defects were not found in this region for all hearts examined. Two hearts additionally showed shortened OFTs that corresponded with unilateral or mixed lineage compositions in the proximal regions. The variable defects in LR cell lineage composition were preceded by increased *Pitx2c* expression in the lateral plate mesoderm, suggesting that dosage and/or duration of laterality gene expression may be just as important as unilateral restriction of expression in modulating LR development. Consistent with this interpretation, an increased susceptibility of the atria to altered *Pitx2* expression has been noted in *Pitx2*-null mice in which hypomorphic alleles can rescue all defects typical of the knockout with the exception of right atrial isomerism, which requires a higher dose of *Pitx2c* expression/activity (Liu et al., 2001). These findings, coupled with results obtained in our experimental situs solitus embryos, indicate that subtle laterality defects can occur in embryos with otherwise normal body situs. This suggests that some incidences of seemingly isolated CHDs, particularly those that occur in individuals whose families are afflicted with some form of heritable laterality disease (Morelli et al., 2001), may in fact represent less obvious manifestations of a LR asymmetry defect. Although important details of the molecular pathway linking LR lineage allocation, *Pitx2c* expression and cardiogenesis remain to be defined, the ability of the LR axis to regulate cell lineage allocation reveals a fundamental and previously unappreciated mechanism by which organ laterality becomes patterned during vertebrate embryogenesis.

We thank Drs Adriana Gittenberger de Groot, Robert Anderson, Chris Drake and Tim McQuinn for helpful discussion; Drs Andy Warkman and Paul Krieg for technical advice; Katherine Chike-Harris for expert technical assistance; and the anonymous reviewers for insightful suggestions. Supported by an American Heart Association Scientist Development Grant and HL73270 (to A.F.R.) and a SC COBRE for Cardiovascular Disease P20-RR-1634 (to R. R. Markwald).

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