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# Conserved regulation and role of *Pitx2* in situs-specific morphogenesis of visceral organs

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*Pitx2* is expressed in developing visceral organs on the left side and is implicated in left-right (LR) asymmetric organogenesis. The asymmetric expression of *Pitx2* is controlled by an intronic enhancer (ASE) that contains multiple Foxh1-binding sites and an Nkx2-binding site. These binding sites are essential and sufficient for asymmetric enhancer activity and are evolutionarily conserved among vertebrates. We now show that mice that lack the ASE of *Pitx2* (*Pitx2*$^{\Delta ASE/\Delta ASE}$ mice) fail to manifest left-sided *Pitx2* expression and exhibit laterality defects in most visceral organs, although the position of the stomach and heart looping remain unaffected. Asymmetric *Pitx2* expression in some domains, such as the common cardinal vein, was found to be induced by Nodal signaling but to be independent of the ASE of *Pitx2*. Expression of *Pitx2* appears to be repressed in a large portion of the heart ventricle and atrioventricular canal of wild-type mice by a negative feedback mechanism at a time when the gene is still expressed in its other domains. Rescue of the early phase of asymmetric *Pitx2* expression in the left lateral plate of *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos was not sufficient to restore normal organogenesis, suggesting that continuous expression of *Pitx2* in the lineage of the left lateral plate is required for situs-specific organogenesis.

**KEY WORDS: Left-right asymmetry, Organogenesis, Pitx2, Mouse**

## INTRODUCTION

Although recent studies have provided insight into how left-right (LR) asymmetry is generated during vertebrate development (Hamada et al., 2002; Levin, 2005; Tabin, 2005), knowledge of this process remains limited. One of many important questions still unanswered concerns the mechanism by which situs-specific organogenesis is executed.

Situs-specific morphogenesis in the mouse begins soon after the loss of asymmetric expression of *Nodal* on the left side of the lateral plate mesoderm (LPM). The looping of the heart tube toward the right side is followed by asymmetric lobe formation in the lungs, rotation of the digestive tract and remodeling of the vascular system. This asymmetric morphogenesis occurs in response to Nodal, which functions as a left-side determinant. Organ primordia that have received the Nodal signal thus adopt a left-side morphology, whereas they adopt a right-side morphology in the absence of this signal. At the cellular level, the left and right sides of organ primordial may differ with regard to rate of cell proliferation, apoptosis or migration. However, the cellular basis of the generation of morphological asymmetry is poorly understood.

The bicoid-type homeobox transcription factor Pitx2 is thought to play a major role in asymmetric morphogenesis (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). The *Pitx2* gene encodes three isoforms: Pitx2a, Pitx2b and Pitx2c. *Pitx2a* and *Pitx2b* mRNAs are generated by alternative splicing and are expressed bilaterally, while *Pitx2c* mRNA, a transcript from an alternative promoter, is expressed asymmetrically (Kitamura et al., 1999; Liu et al., 2002; Schweickert et al., 2000). *Pitx2* knockout mice manifest LR defects in many organs, including the typical right isomerism of the lungs (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). However, the exact role of Pitx2 in situs-specific organogenesis is unknown.

Asymmetric expression of *Pitx2* begins in the left LPM concomitantly with that of *Nodal*, but it persists after *Nodal* expression disappears. *Pitx2* expression is thus still apparent in the LPM-derived mesenchyme of various visceral organs at the late somite stage. Asymmetric expression of mouse *Pitx2* is conferred by an enhancer (ASE) that contains three binding sites for the transcription factor Foxh1, a target of Nodal signaling, as well as a binding site for the homeobox transcription factor Nkx2 (Shiratori et al., 2001). The Foxh1 binding sites are essential for the initiation of asymmetric *Pitx2* expression, whereas the Nkx2-binding site is dispensable for such initiation but necessary for maintenance of late-stage expression. The left-sided expression of *Pitx2* is thus initiated by Nodal signaling and maintained by Nkx2. Consistently, asymmetric *Pitx2* expression is lost in mutant mice lacking any of the Nodal signaling components, such as Foxh1 (Yamamoto et al., 2003) and cryptic [Cfc1 – Mouse Genome Informatics; a co-receptor for Nodal (Shen and Schier, 2000; Yan et al., 1999)]. The LR asymmetric expression pattern of *Pitx2* is conserved among all vertebrates examined.

We have now studied the regulation and role of *Pitx2* in asymmetric organogenesis by examining the asymmetric enhancer ASE of this gene. Our data suggest that asymmetric *Pitx2* expression in vertebrates other than the mouse is also regulated by this highly conserved enhancer. Generation of mice that lack the *Pitx2* ASE confirmed its essential role in regulation of *Pitx2* and revealed the precise role of Pitx2 in situs-specific morphogenesis. We also tested the importance of two-step regulation of *Pitx2* expression by rescue experiments with a *Pitx2* transgene.

## MATERIALS AND METHODS

### Transgenic mouse assay

Genomic DNA libraries for rat, human and zebrafish (Stratagene) or for chicken (Clontech) were screened by hybridization with mouse *Pitx2* cDNA as a probe. DNA fragments containing the last intron of *Pitx2* were obtained

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from the isolated clones by PCR with a pair of primers corresponding to exons 4 and 5. DNA fragments containing the whole intron 5 of vertebrate *Pitx2* genes were ligated to the 5′ end of an *hsp68-lacZ* reporter construct (Kothary et al., 1988). Seven tandem repeats of the Nkx2-binding sequence of mouse *Pitx2* were ligated to the 5′ end of a 380 bp DNA fragment containing the ASE of mouse *Lefty2* (Saijoh et al., 1999) and to the *hsp68-lacZ* construct. A *Foxh1-lacZ* transgene was constructed from a 234 kb bacterial artificial chromosome (BAC) clone (RP23-156P23) containing mouse *Foxh1*; an internal ribosomal entry site (IRES)-*lacZ* cassette was thus inserted into the 3′ untranslated region of *Foxh1* upstream of the poly(A) addition sequence in the BAC clone. Transgenic mice were generated by injection of these various *lacZ* constructs into the pronucleus of fertilized eggs as described previously (Saijoh et al., 1999). They were then collected at embryonic day (E) 8.2 or 10.5 and stained with the β-galactosidase substrate X-gal.

### Generation of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice

A targeting vector was designed to delete the 0.6 kb ASE region of *Pitx2* (see Fig. 4A). To delete the *PGK-neo* sequence, we subjected targeted embryonic stem (ES) cells to electroporation with a *CAG-Cre* vector (Sakai and Miyazaki, 1997). ES cell culture and injection of blastocysts obtained by crossing B6C3F1 males and C57BL/6J females were performed by standard methods (Hogan et al., 1994). Male chimeras were bred with C57BL/6J females to yield heterozygous $F_1$ offspring, which were then mated with each other to produce *Pitx2*$^{\Delta ASE/\Delta ASE}$ homozygotes. Offspring and embryos were genotyped by PCR; primers for the wild-type *Pitx2* allele were 5′-TCACTGAAATCCCTGGGGAGA (ΔASE55) and 5′-AGAAGCA-AGCCTCACGCACTT (ΔASE53), and those for the targeted allele wereΔASE55 and 5′-AGCCACAGAAGCCATCAACTT [ΔASE53(2)]. The former primers yielded a 134 bp product and the latter a 370 bp product.

### Whole-mount in-situ hybridization and histological analysis

Whole-mount in-situ hybridization was performed with standard protocols and probes specific for *Pitx2* (Yoshioka et al., 1998) or cryptic (Shen et al., 1997) mRNAs. For histological analysis, some embryos that were subjected to X-gal staining or whole-mount in-situ hybridization were embedded in paraffin and sectioned at a thickness of 8 μm. For scanning electron microscopy, E12.5 mouse embryos were processed using the standard procedure (Nonaka et al., 2005) and were observed using a scanning electron microscope (S-2600N, Hitachi, Japan).

### Rescue by *Pitx2* transgenes

Two transgenes that confer asymmetric expression of *Pitx2c* were designed as follows. A 0.9-kb DNA fragment containing the wild-type ASE of *Pitx2* or a mutant ASE (NmASE) in which the Nkx2 binding site was mutated was linked to a 1.3-kb fragment of the *Pitx2c* promoter, *Pitx2c* cDNA, and an IRES-*lacZ* cassette. The resulting constructs were microinjected into the pronucleus of fertilized mouse eggs obtained by crossing *Pitx2*$^{\Delta ASE/+}$ heterozygous B6/129 males and wild-type C57BL/6J females. Thirteen and 10 lines were established for the wild-type and mutant ASE constructs, respectively. Expression of each transgene was monitored by X-gal staining. Three transgenic lines (*Tg39* and *Tg55* for the wild-type ASE, and *Tg50* for the mutant ASE) that showed similar levels of transgene expression at E8.2 were studied. *Pitx2*$^{\Delta ASE/+}$ males harboring each transgene were mated with *Pitx2*$^{\Delta ASE/+}$ females to generate *Pitx2*$^{\Delta ASE/\Delta ASE}$ homozygotes with the transgene.

### Cell lineage analysis

Two permanent transgenic mouse lines, *Pitx2 17-lacZ* and *Pitx2-Cre*, were established. The *Pitx2 17-lacZ* and *Pitx2-Cre* transgenes consist of a 1.3 kb fragment of the *Pitx2c* promoter and either *lacZ* or *Cre* followed by a 17 kb fragment containing exons 1c, 4 and 5, as well as the ASE and 3′ flanking region of *Pitx2* (Shiratori et al., 2001). These transgenes were first introduced into *Pitx2*$^{\Delta ASE/+}$ heterozygotes. To study the lineages of left LPM cells expressing *Pitx2*, we crossed *Pitx2*$^{\Delta ASE/+}$ males harboring the *Pitx2-Cre* transgene with *Pitx2*$^{\Delta ASE/+}$ females harboring the *CAG-CAT-lacZ* transgene (Sakai and Miyazaki, 1997). The resulting embryos were collected at E9.5 and stained with X-gal.

## RESULTS

### Conserved role of the asymmetric enhancer ASE in *Pitx2* regulation among vertebrates

Asymmetric expression of mouse *Pitx2* is controlled by an enhancer (ASE) located in the last intron, intron 5 (Shiratori et al., 2001). The ASE contains three Foxh1-binding sequences and an Nkx2-binding sequence (Fig. 1). The former sites are required for initiation of *Pitx2* expression in left LPM at E8.2 (Fig. 2A), whereas the latter is responsible for maintenance of asymmetric expression from E9.5 to 10.5 (Fig. 2F,K,P,U) (Shiratori et al., 2001). We therefore examined *Pitx2* genes of other vertebrates to determine whether this transcriptional regulatory mechanism for achieving asymmetric expression is conserved.

We isolated *Pitx2* genes from human, rat, chicken and zebrafish. For chicken *Pitx2*, a 16 kb region containing the last intron, which is located between the exons encoding the homeodomain in all vertebrates examined, was tested for ASE activity with a transgenic mouse assay. ASE activity was initially localized to a 9.0 kb region and was subsequently mapped to a 2.0 kb region containing the last intron. An *hsp68-lacZ* reporter construct driven by the 9.0 kb fragment of chicken *Pitx2* thus gave rise to asymmetric X-gal staining not only in left LPM at E8.2 (Fig. 2C) but also in various primordial organs, including the common atrial chamber, lung bud, septum transversum and gut dorsal mesentery at E10.5 (Fig. 2H,M,R,W). Nucleotide sequencing of the 2.0 kb region containing intron 5 revealed the presence of two Foxh1-binding sequences and an Nkx2-binding sequence in the last intron (Fig. 1). For the *Pitx2* genes of human, rat and zebrafish, DNA fragments containing the entire last intron were isolated by PCR and sequenced. Each fragment contained two or three Foxh1-binding sequences and an Nkx2-binding sequence (Fig. 1). The fragment of human *Pitx2* showed typical ASE activity, with the corresponding *hsp68-lacZ* transgene giving rise to left-sided X-gal staining in LPM at E8.2 (Fig. 2B) as well as in various organs at E10.5 (Fig. 2G,L,Q,V). The DNA fragment containing the last intron of zebrafish *Pitx2* conferred asymmetric expression of the reporter construct in left LPM at E8.2 (Fig. 2E) but not in primordial organs at E10.5 (Fig. 2J,O,T,Y). Moreover, expression of the transgene in left LPM was apparent

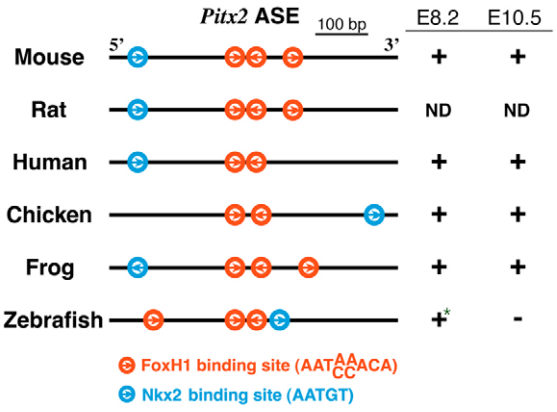


**Fig. 1. Conservation of an asymmetric enhancer (ASE) of *Pitx2* among vertebrates.** Structures of the *Pitx2* ASE from various vertebrates (located in the last intron of *Pitx2*) are shown on the left. Red and blue circles represent Foxh1- and Nkx2-binding sites, respectively, with the arrows indicating their orientation. The enhancer activity of each ASE in transgenic mouse assays at E8.2 and 10.5 is summarized on the right. The ASE of zebrafish *Pitx2* possesses activity only in the anterior portion of left LPM (asterisk). ND, not determined.

DEVELOPMENT

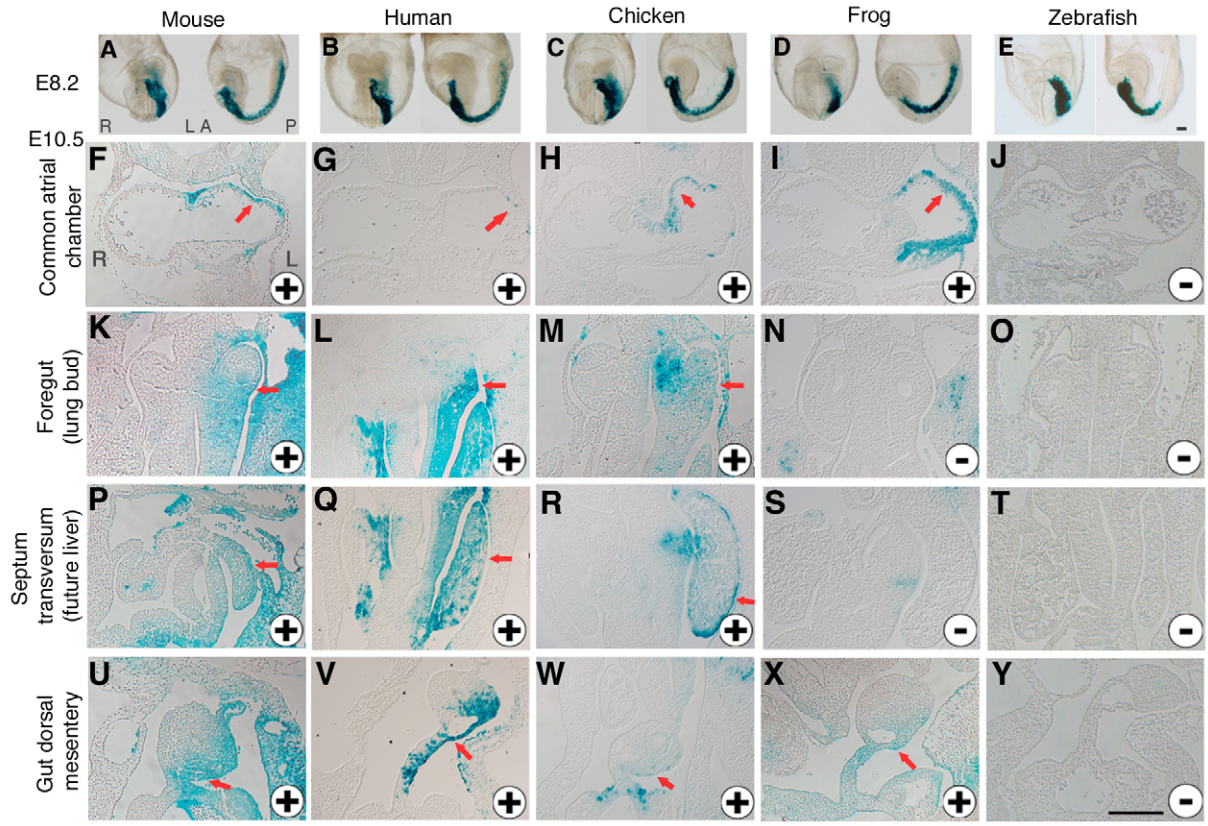


**Fig. 2. Activity of the *Pitx2* ASE from various vertebrates.** Reporter constructs (*hsp68-lacZ*) driven by the *Pitx2* ASE from various vertebrates were injected into fertilized mouse eggs, and the resulting embryos were stained with X-gal. (**A-E**) Frontal and lateral views of transgenic mouse embryos at E8.2. All constructs examined gave rise to asymmetric X-gal staining in left LPM, although the zebrafish ASE was active only in the anterior portion of left LPM. (**F-Y**) Transverse sections of embryos at E10.5. Activities in the common atrial chamber (F-J), foregut (lung bud) (K-O), septum transversum (future liver) (P-T), and gut dorsal mesentery (U-Y) are shown. Red arrows indicate the domains in which the ASE was specifically active. Encircled + or – signs indicate whether the ASE was active or inactive, respectively. The ASE from mouse, human or chicken *Pitx2* showed left-sided activity in the indicated organs. The frog ASE was active in the common atrial chamber and gut dorsal mesentery, but inactive in the foregut and septum transversum. The zebrafish ASE was inactive in all organs. The number of the embryos examined and variations in expression patterns are shown in Table S1 of the supplementary material. Scale bars: 200 μm. A, anterior; L, left; P, posterior; R, right.

only in the anterior portion (Fig. 2E). As we showed previously (Shiratori et al., 2001), the ASE of frog *Pitx2* was active in left LPM at E8.2 (Fig. 2D) as well as in the common atrial chamber and gut dorsal mesentery but not in the lung bud or septum transversum at E10.5 (Fig. 2I,N,S,X). These results thus showed that an enhancer that regulates asymmetric expression of *Pitx2* (ASE) is evolutionarily conserved among vertebrates. In all species examined, the ASE contains multiple Foxh1-binding sites and an Nkx2-binding site, suggesting that asymmetric expression of vertebrate *Pitx2* is initiated by Nodal signaling and maintained by Nkx2.

The role of the Nkx2-binding sequence in the *Pitx2* ASE was examined further by linking it to the asymmetric enhancer (ASE) of mouse *Lefty2*. The *Lefty2* ASE contains two Foxh1-binding sequences but no Nkx2-binding sequence, and it gives rise to asymmetric expression in left LPM at E8.2 (Fig. 3A) (Saijoh et al., 1999) but not in organ primordia at E10.5 (Fig. 3C,E,G) (Shiratori et al., 2001). However, the addition of seven copies of the Nkx2-binding sequence of mouse *Pitx2* to the *Lefty2* ASE (*$N_7$-Lefty2 ASE*) (Fig. 3B) resulted in asymmetric expression of the corresponding *hsp68-lacZ* reporter construct in various visceral organs at E10.5 (Fig. 3D,F,H). An Nkx2-binding sequence is thus not only essential but also sufficient for maintenance of asymmetric gene expression.

## Loss of LR asymmetric *Pitx2* expression and impairment of situs-specific morphogenesis of many but not all organs induced by ASE deletion

To establish the role of the ASE in *Pitx2* regulation, we generated a mutant *Pitx2* allele that lacks the ASE (*$Pitx2^{\Delta ASE}$*) (Fig. 4). We first examined how asymmetric *Pitx2* expression was affected by ASE deletion. *$Pitx2^{\Delta ASE/\Delta ASE}$* embryos developed normally until the early somite stage. As expected, asymmetric *Pitx2* expression in left LPM was abolished at the early somite stage, whereas bilateral *Pitx2* expression in the head mesenchyme remained unaffected (Fig. 5A,B). At later stages (E9.0 to 9.5), left-sided *Pitx2* expression in various primordial organs also did not develop in *$Pitx2^{\Delta ASE/\Delta ASE}$* embryos (Fig. 5D,E,G,H,J,K,M,N,P,Q). These results thus showed that asymmetric expression of *Pitx2* was lost as a result of specific deletion of the ASE, confirming the role of the ASE in such expression.

*$Pitx2^{\Delta ASE/\Delta ASE}$* newborn mice manifested various LR defects, including those previously reported for *Pitx2* null mice (Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999) and mutant mice specifically lacking *Pitx2c* (Liu et al., 2002). Thus, *$Pitx2^{\Delta ASE/\Delta ASE}$* mice showed right isomerism in the lungs (Fig. 6A,B; 18/18 mice examined), reversed positioning of the great arteries (Fig. 6C,D; 18/18), reversed positioning of the heart apex toward the right (Fig.

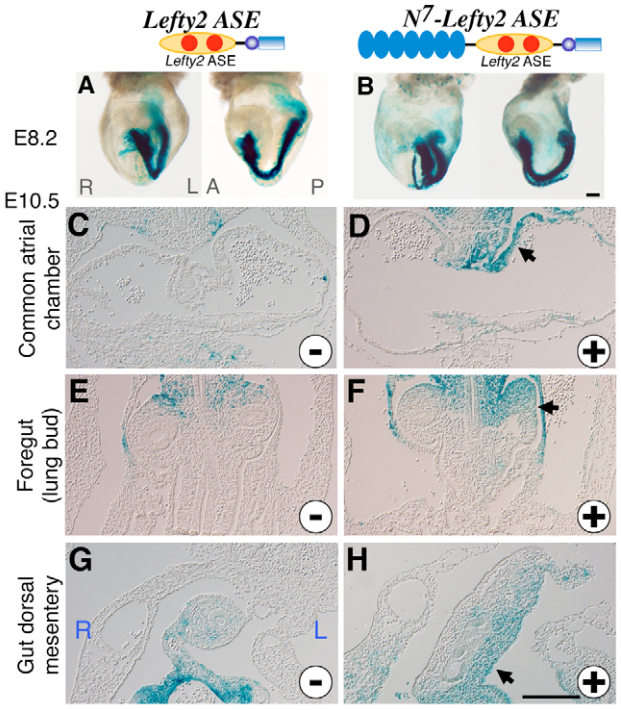


**Fig. 3. Prolongation of the transient enhancer activity of the ASE of *Lefty2* by addition of Nkx2-binding sequences.** (**A**,**B**) Structures of two *lacZ* transgenes are shown at the top. Red circles and blue ovals indicate Foxh1- and Nkx2-binding sequences, respectively. The partially blue circles and boxes indicate the *hsp68* promoter and *lacZ*, respectively. The construct in A includes the *Lefty2* ASE, which contains two Foxh1-binding sequences. The construct in B contains the *Lefty2* ASE plus seven tandem repeats of the Nkx2-binding sequence derived from the *Pitx2* ASE. X-gal staining patterns of E8.2 mouse embryos harboring each transgene are shown below. Both *lacZ* constructs gave rise to typical asymmetric staining in left LPM. (**C-H**) Transverse sections of X-gal-stained transgenic embryos at E10.5. Expression of *lacZ* is shown for the common atrial chamber (C,D), foregut (E,F), and gut dorsal mesentery (G,H). Black arrows indicate the domains where asymmetric X-gal staining was detected. Encircled + or – signs indicate whether the enhancer was active or inactive, respectively. The *Lefty2 ASE* construct did not give rise to asymmetric staining (C,E,G), whereas the *N7-Lefty2 ASE* construct did so in the common cardinal vein, lung bud and gut dorsal mesentery (D,F,H). Scale bars: 200 μm.

6C,D; 5/18), bilateral inferior vena cava (Fig. 6C,D; 5/18), right isomerism in the atrium (Fig. 6E,F; 18/18), and an endocardial cushion defect, as well as a common atrioventricular (AV) valve with a ventricular septal defect (VSD) and atrial septal defect (ASD) (Fig. 6G,H; 4/4), and double-outlet right ventricle (DORV). To know the cellular basis of the endocardial cushion defects, we also examined the AV cushions at E12.5 by scanning electron microscope (Fig. 6I,J) and by sagittal sectioning (Fig. 6K,L). The AV cushions consist of two portions, the superior AV cushion (SAVC) and inferior AV cushion (IAVC). The SAVC and IAVC are fused with each other at E12.5 in the wild-type embryo (Fig. 6I,K), but they remained separated in the $Pitx2^{\Delta ASE/\Delta ASE}$ embryo (Fig. 6J,L). In the mutant embryo, SAVC was hypoplastic while IAVC appeared relatively normal in size (Fig. 6J,L). These results suggest that a hypoplastic SAVC is responsible for the common AV valve with VSD and ASD in $Pitx2^{\Delta ASE/\Delta ASE}$ mice. This is consistent with the fact that Pitx2 is expressed in the myocardium adjacent to SAVC but is largely absent in the myocardium adjacent to IAVC (Kitamura et al., 1999).

We also noted previously unrecognized LR defects in the $Pitx2^{\Delta ASE/\Delta ASE}$ mice, including reversed positioning of the azygos vein (12/18) and aorta (9/18) on the right side of the thorax (Fig.

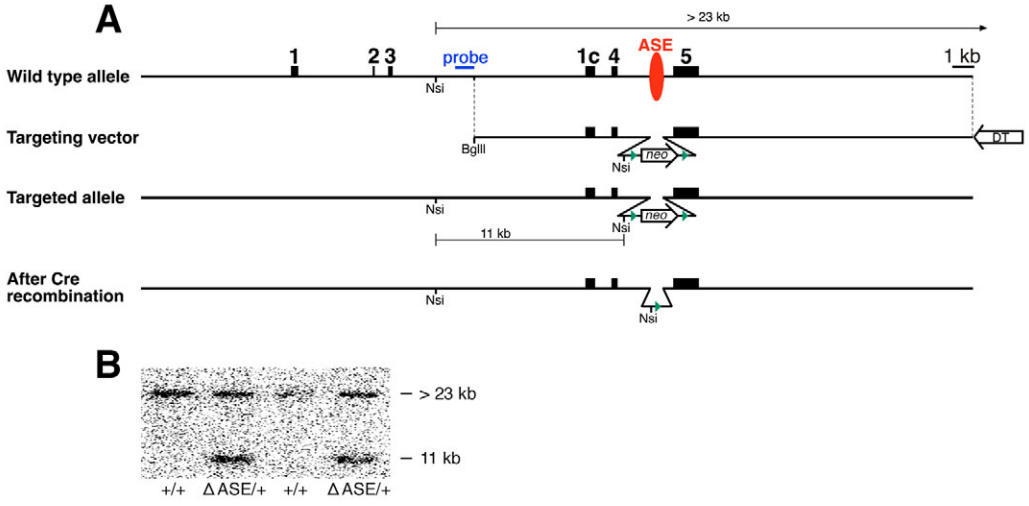


**Fig. 4. Generation of mice that lack the ASE of *Pitx2*.** (**A**) Schematic representation of the mouse wild-type *Pitx2* allele, a targeting vector designed to delete the 0.6 kb region of *Pitx2* that contains the ASE (red oval), as well as the targeted allele before and after Cre-mediated recombination. *Pitx2* contains six exons (black boxes) and two promoters, with transcription beginning at exon 1 or exon 1c. (**B**) Southern blot analysis of Nsi-digested DNA from ES cells of the indicated *Pitx2* genotypes before Cre-mediated recombination. The probe used for hybridization is shown by the blue bar on the top. The wild-type and mutant alleles give rise to hybridizing fragments of >23 and 11 kb, respectively. Green triangles, loxP sequences; *neo*, neomycin resistance gene under the control of the phosphoglycerate kinase gene promoter; DT, diphtheria toxin gene.

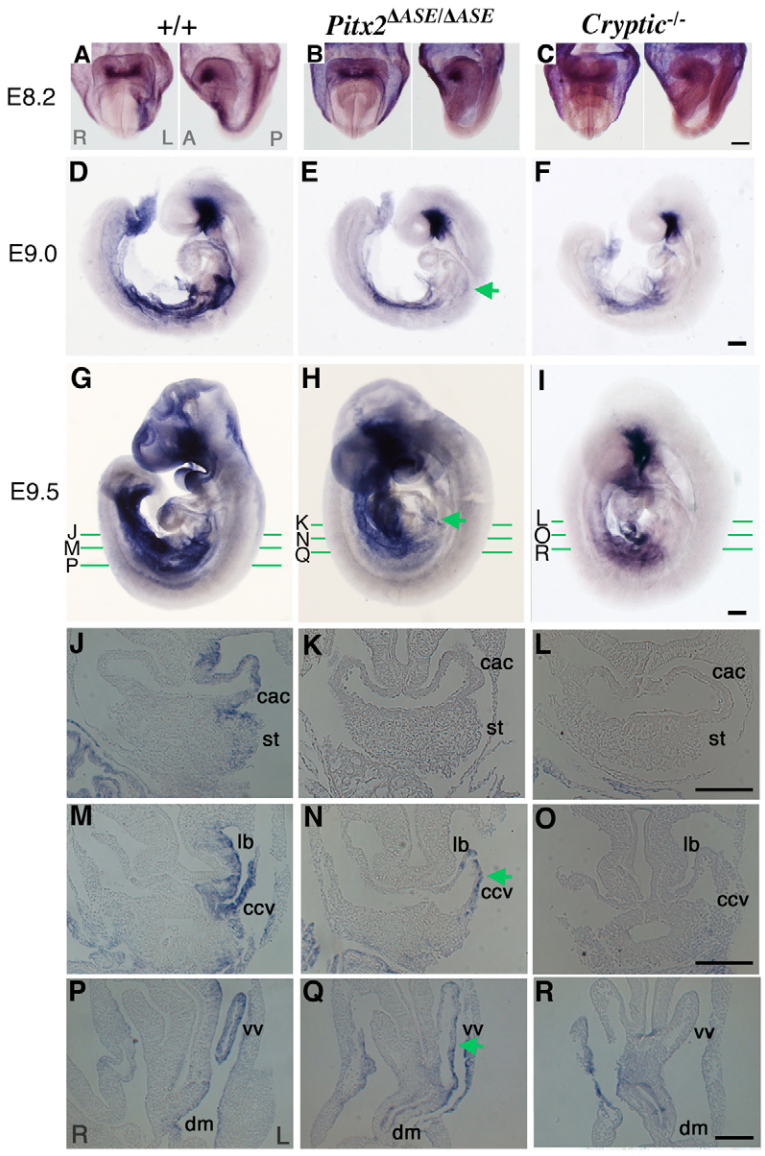


**Fig. 5. Loss of most LR asymmetric expression domains of *Pitx2* in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos.** Expression of *Pitx2* was examined in wild-type (A,D,G,J,M,P), *Pitx2*$^{\Delta ASE/\Delta ASE}$ (B,E,H,K,N,Q) or cryptic$^{-/-}$ (C,F,I,L,O,R) embryos at E8.2 (**A-C**), E9.0 (**D-F**) or E9.5 (**G-R**) by whole-mount in-situ hybridization. The whole-mount embryos shown in G-I were sectioned transversely at the indicated planes and the sections are shown in J-R. Expression of *Pitx2* in left LPM at E8.2 was lost in *Pitx2*$^{\Delta ASE/\Delta ASE}$ and cryptic$^{-/-}$ embryos. Asymmetric *Pitx2* expression was lost in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos at E9.0, with the exception of a reduced level of expression remaining in the common cardinal vein and vitelline vein (green arrow in E). Asymmetric *Pitx2* expression was completely absent from cryptic$^{-/-}$ embryos at E9.0. Asymmetric *Pitx2* expression remained apparent at a reduced level in the common cardinal vein and vitelline vein of *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos at E9.5 (green arrows in H, N and Q) but was absent from cryptic$^{-/-}$ embryos at this time. Scale bars: 200 μm. cac, common atrial chamber; ccv, common cardinal vein; dm, gut dorsal mesentery; lb, lung bud; st, septum transversum; vv, vitelline vein.

6M,N), reversed relation of the aorta and vena cava in the abdomen (Fig. 6U,V; 4/16), abnormal location of the portal vein on the ventral side of the duodenum (Fig. 6O,P; 1/11), abnormal rotation of the gut, such as aberrant looping of the duodenum (Fig. 6Q,R; 12/12), and the absence of a cross of the duodenum and colon (Fig. 6S,T; 11/12), and mal-location of the pancreas on the ventral or right side of the duodenum (Fig. 6R; 12/12).

These morphological defects of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice resemble those of cryptic knockout mice (Yan et al., 1999) and of conditional mutant mice that lack *Foxh1* expression in the lateral plate (Yamamoto et al., 2003). Unlike the cryptic or *Foxh1* mutant animals, however, some laterality-dependent events remained unaffected in *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. In particular, the directions of heart looping and embryonic turning were normal in all (12/12) *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos examined. Furthermore, the stomach was always located on the left side (12/12) and the position of the spleen was normal (12/12), although the latter was reduced in size (12/12), as has been described for *Pitx2* knockout mice (Lin et al., 1999; Lu et al., 1999). These events and organ positions are thus probably regulated by a mechanism dependent on Nodal-cryptic-Foxh1 signaling but independent of the Pitx2 ASE.

## Expression domains of *Pitx2* that are independent of the ASE but dependent on cryptic and Foxh1

In *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice, asymmetric expression of *Pitx2* was not detected in left LPM at E8.2 (Fig. 5B) and most asymmetric expression domains normally apparent at E9.0 to 9.5 were also lost (Fig. 5E,H,K,N,Q). A reduced but substantial level of asymmetric *Pitx2* expression was detected in the common cardinal vein and vitelline vein of the mutant embryos at E9.0 to 9.5, however (Fig. 5E,H,N,Q). To determine the mechanism by which this ASE-independent expression of *Pitx2* is regulated, in particular whether or not it is induced by Nodal, we examined cryptic$^{-/-}$ mice (Yan et al., 1999) and *Foxh1* conditional mutant mice (Yamamoto et al., 2003) for *Pitx2* expression in these veins. In the cryptic$^{-/-}$ embryos, asymmetric expression of *Pitx2* was lost in all organs, including the common cardinal vein and vitelline vein, at E9.0 and 9.5 (Fig. 5F,I,L,O,R). These expression domains were also lost in the *Foxh1* mutant (Yamamoto et al., 2003). The frequencies of an abnormal phenotype for the inferior vena cava in the thorax and for the relation between the portal vein and duodenum were 28 and 9%, respectively, in *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice, compared with corresponding values of 68 and 47% for cryptic$^{-/-}$ mice (data not shown). The

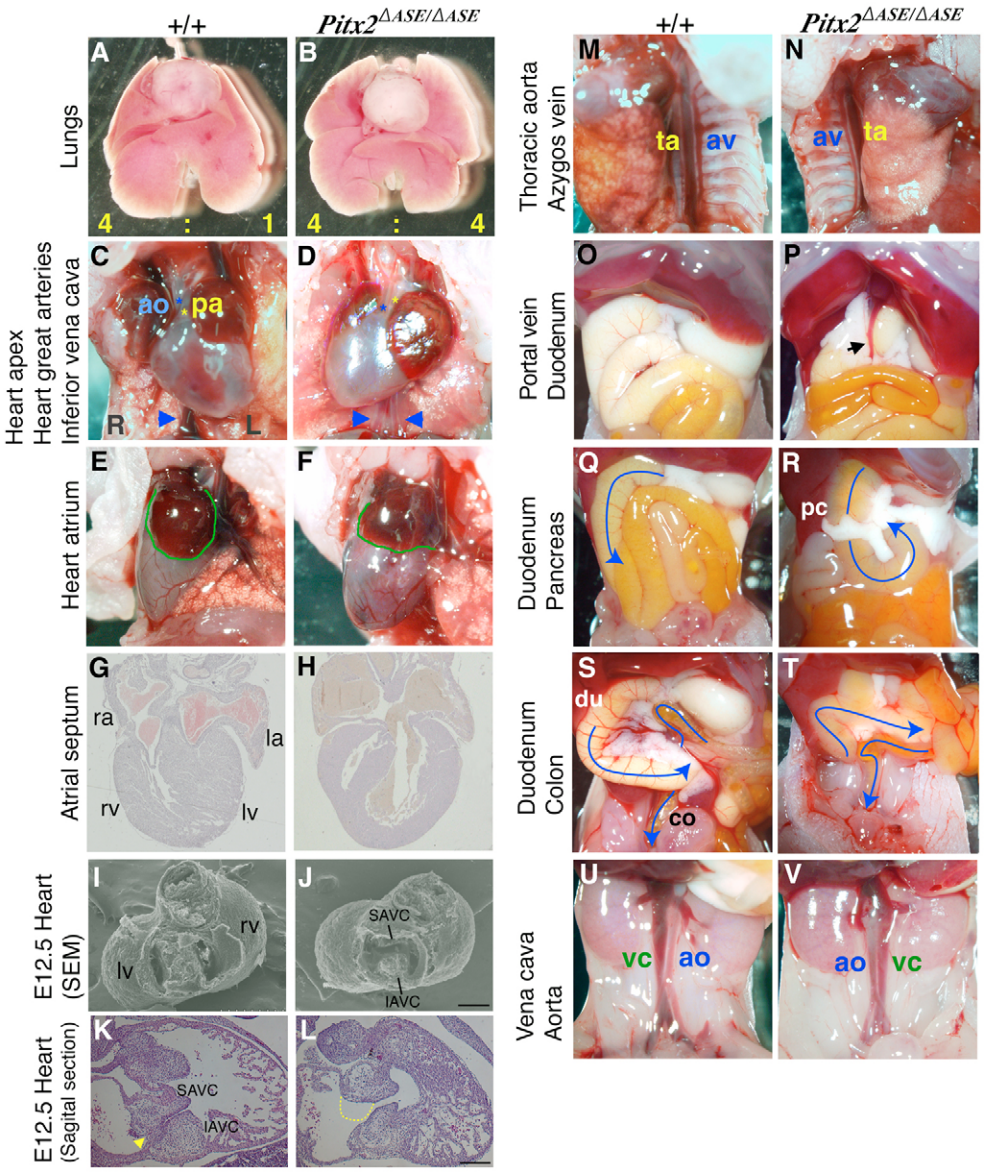


**Fig. 6. LR defects in *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice.** Visceral organs and heart sections of wild-type (+/+) and *Pitx2*$^{\Delta ASE/\Delta ASE}$ neonates are shown in A-H,M-V. (**A**,**B**) The left and right lungs of wild-type mice have one and four lobes (indicated in yellow), respectively, whereas the lungs of all *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice had four lobes on each side. (**C**,**D**) The relative positions of the aorta (ao, blue asterisk) and pulmonary artery (pa, yellow asterisk) are reversed in *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. The position of the heart apex is toward the left in wild-type mice but was reversed (toward the right) in 28% of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. The inferior vena cava is present only on the right side of the thorax in wild-type mice (C, blue arrowhead) but was bilateral in 28% of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice (D, two arrowheads). (**E**,**F**) External morphology of the atrium showing right isomerism in *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. In wild-type mice, the left atrium has a narrow junction to the venous component (green line in E), whereas the right atrium has a wide junction. In *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice, both the left (green line in F) and right atria exhibited a morphology similar to that of the right atrium of the wild type. (**G**,**H**) Frontal sections of the heart. Atrial septal defect was apparent in the mutant heart. (**I-L**) AV cushions of the heart at E12.5 are shown by scanning electron microscopy (SEM) (I,J) and by sagittal sectioning of HE-stained hearts (K,L). SAVC and IAVC are fused with each other in the wild-type heart, but they remain separated in the *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos. The yellow arrowheads in K denote the boundary between SAVC and IAVC. The dotted area in L indicates a portion of SAVC that is missing in the *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos. (**M**,**N**) The azygos vein and thoracic aorta are located on the left side of the thorax in wild-type mice, whereas they were present on the right side in some *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. (**O**,**P**) The portal vein passes dorsally to the duodenum and is not visible from the ventral side in wild-type mice, but it was on the ventral side of the duodenum in some *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice (black arrow in P). (**Q-T**) Abnormal rotation of the gut, including aberrant looping of the duodenum (S) or the lack of a cross of the duodenum and colon (T), was apparent in *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice at a high frequency. Blue arrows indicate positioning of the gut. The pancreas, which is located on the left side of the duodenum and is located behind the ileum in a ventral view in wild-type mice (Q), was positioned on the ventral or right side of the duodenum in *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice (R). (**U**,**V**) In the abdomen of wild-type mice, the aorta and vena cava are located on the left and right sides of the midline, respectively; their relative positions were reversed in 25% of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. Scale bars: 200 μm. ao, aorta; az, azygos vein; co, colon; du, duodenum; IAVC, inferior atrioventricular cushion; la, left atrium; lv, left ventricle; pc, pancreas; ra, right atrium; rv, right ventricle; SAVC, superior atrioventricular cushion; ta, thoracic aorta; vc, vena cava.

inferior vena cava and portal vein are derived from the common cardinal vein and vitelline vein, respectively, in both of which a reduced level of asymmetric *Pitx2* expression remained in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos. These results suggest that the *Pitx2* expression in these veins of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice is also induced by Nodal signaling.

We also examined whether cryptic, *Foxh1* and *Nodal* are expressed in the ASE-independent expression domains of *Pitx2*. cryptic expression, as revealed by whole-mount in-situ hybridization, was apparent in LPM at E8.2 (Fig. 7A) but not in the common cardinal vein or vitelline vein at E9.0 or 9.5 (Fig. 7D,G,J,M,P,S). To detect expression of *Foxh1* or *Nodal*, we examined the *Foxh1-lacZ* BAC transgenic mice (see Materials and methods) and *Nodal*$^{lacZ/+}$ mice (Collignon et al., 1996), respectively. The *Foxh1-lacZ* BAC transgene was expressed in left LPM at E8.2 (Fig. 7C), consistent with the distribution of *Foxh1* mRNA revealed by whole-mount in-situ hybridization (Saijoh et al., 2000). Expression of the *Foxh1-lacZ* transgene was apparent in the heart at E9.0 and 9.5, where *Pitx2* is expressed, but it was absent from the common cardinal vein and vitelline vein (Fig. 7F,I,L,O,R,U).

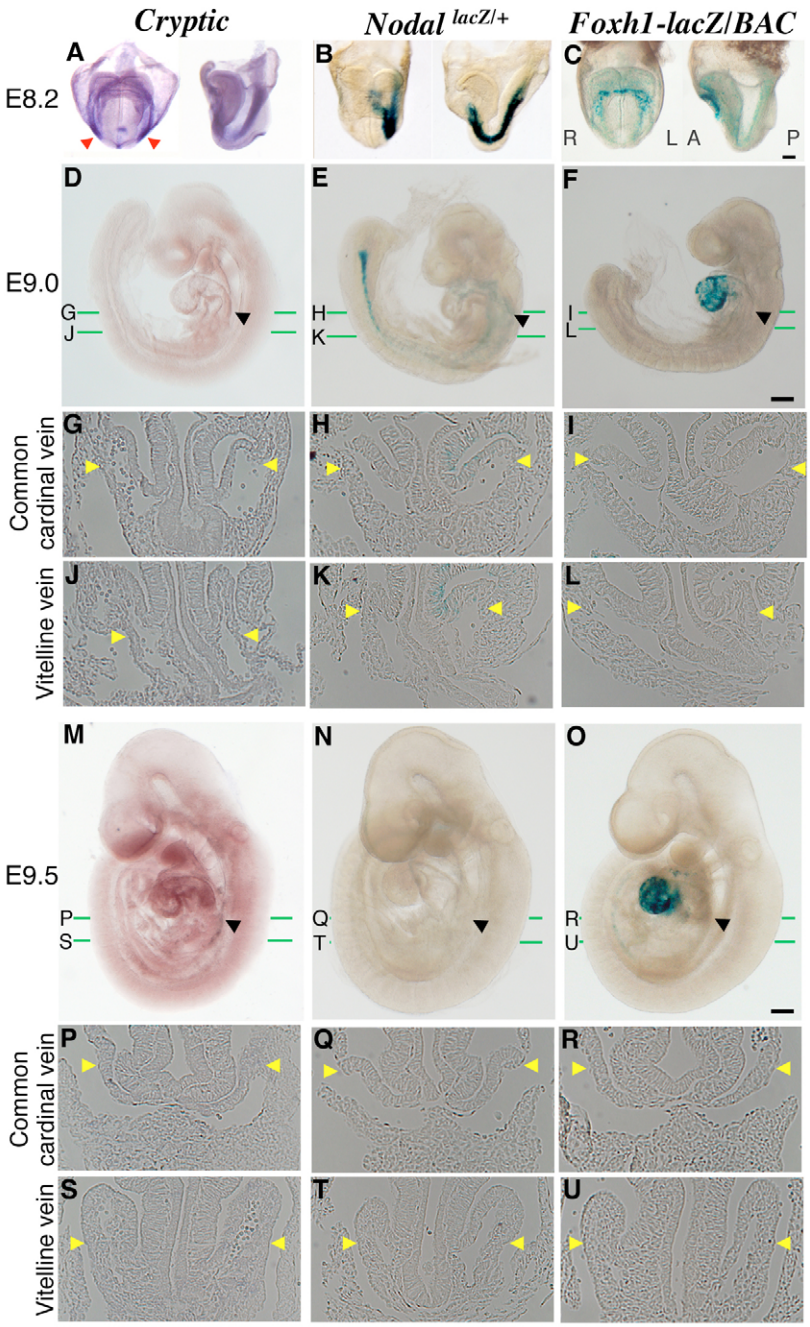


**Fig. 7. Expression patterns of genes related to Nodal signaling.** (**A**,**D**,**G**,**J**,**M**,**P**,**S**) Expression of cryptic was examined in wild-type mouse embryos by whole-mount in-situ hybridization. Expression was bilateral in LPM at E8.2 (red arrowheads in A) but was not detected at E9.0 (D,G,J) or E9.5 (M,P,S). (**B**,**E**,**H**,**K**,**N**,**Q**,**T**) *Nodal* expression was examined by X-gal staining of *Nodal*$^{lacZ/+}$ embryos. Staining was apparent in left LPM at E8.2 (B), was detected at a low level in the heart region containing the common cardinal vein and vitelline vein at E9.0 (E,H,K) and was not observed at E9.5 (N,Q,T). (**C**,**F**,**I**,**L**,**O**,**R**,**U**) *Foxh1* expression was examined by X-gal staining of embryos expressing a *Foxh1-lacZ* transgene. Staining was apparent in LPM and heart primordial cells at E8.2 (C) and was evident in the heart, but not in the common cardinal vein and vitelline vein, at E9.0 (F,I,L) and E9.5 (O,R,U). Transverse sections were prepared after whole-mount in-situ hybridization (G,J,P,S) or X-gal staining (H,I,K,L,Q,R,T,U). Black and yellow arrowheads in D-U indicate the region that corresponds to the common cardinal vein and vitelline vein and in which asymmetric *Pitx2* expression persists in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos. Scale bars: 200 μm.

Similarly, *Nodal* expression was apparent in left LPM at E8.2 (Fig. 7B), but it was detected at only a low level at E9.0 (Fig. 7E,H,K) and not at all at E9.5 (Fig. 7N,Q,T) in the common cardinal vein and vitelline vein.

These results suggest that asymmetric expression of *Pitx2* in the common cardinal vein and vitelline vein is induced by Nodal signal and is subsequently maintained in the absence of Nodal signal, which is consistent with our previous observations that asymmetric expression in various organs is maintained by Nkx2 in the absence of Nodal signal (Shiratori et al., 2001).

## Negative feedback regulation by Pitx2

To study the fates of *Pitx2*-expressing cells, we established transgenic mice that express *Cre* under the control of a 17 kb region of *Pitx2* that contains the ASE (Shiratori et al., 2001). These *Pitx2-Cre* mice were crossed with *CAG-CAT-lacZ* mice, which harbor a Cre-responsive *lacZ* transgene (Sakai and Miyazaki, 1997), and the resulting embryos were stained with X-gal. Stained cells were specifically located in the left LPM at E8.2 and on the left side of various visceral organs at E9.5 (data not shown). Given that the ASE is continuously active in left LPM-derived cells between E8.2 and 9.5, the *Pitx2-Cre* transgene would be expected to detect all the cells in which the ASE was once active.

The X-gal staining pattern at E9.5 obtained with the *Pitx2-Cre* transgene was compared with that obtained with *Pitx2 17-lacZ*, which consists of *lacZ* under the control of the 17 kb region of *Pitx2* and would be expected to mark only those cells in which the ASE was active at the time examined. Although the two staining patterns were highly similar, differences were detected in the heart ventricle and AV canal. A large portion of the ventricle and myocardium

adjacent to the IAVC of the AV canal was X-gal negative for *Pitx2 17-lacZ* (Fig. 8A-D) but X-gal positive for *Pitx2-Cre* (Fig. 8I-L). This differentially stained region thus represents a domain in which *Pitx2* expression is induced at E8.2 but is repressed by E9.5, earlier than the remaining expression domains are repressed.

The X-gal staining pattern yielded by *Pitx2 17-lacZ* in $Pitx2^{\Delta ASE/\Delta ASE}$ embryos at E9.5 (Fig. 8E-H), however, was virtually identical to that conferred by *Pitx2-Cre* in wild-type embryos (Fig. 8I-L). The large portion of the ventricle and AV canal in which *Pitx2 17-lacZ* was not expressed in wild-type embryos was thus X-gal positive in $Pitx2^{\Delta ASE/\Delta ASE}$ embryos harboring this transgene. Such expansion of X-gal-positive cells in $Pitx2^{\Delta ASE/\Delta ASE}$ embryos might result from either an abnormal contribution of cells in which the ASE was once active or from the loss of negative feedback by Pitx2 itself. To distinguish between these possibilities, we examined the pattern of X-gal staining conferred by *Pitx2-Cre* in $Pitx2^{\Delta ASE/\Delta ASE}$ embryos (Fig. 8M-P). The staining pattern was indistinguishable from that of wild-type embryos harboring the same transgene (Fig. 8I-L), which favors the latter possibility. These results are in principle consistent with the fate mapping data in *Xenopus* (Ramsdell et al., 2005) but suggest a negative feedback loop in a portion of the AV canal. However, our findings require further work because a transgene does not always recapitulate correct expression patterns of a gene [as illustrated in Table 1 and the previous reports; for example, Mortlock et al. (Mortlock et al., 2003)].

## Requirement for continuous expression of *Pitx2* in situs-specific morphogenesis

Whereas asymmetric expression of *Nodal* is transient, asymmetric *Pitx2* expression induced in LPM at E8.2 is maintained in LPM-derived cells of visceral organs for an additional 2 days. We next examined whether the earlier expression of *Pitx2* in LPM is sufficient for situs-specific organogenesis of some organs or whether continuous expression is necessary for all organs.

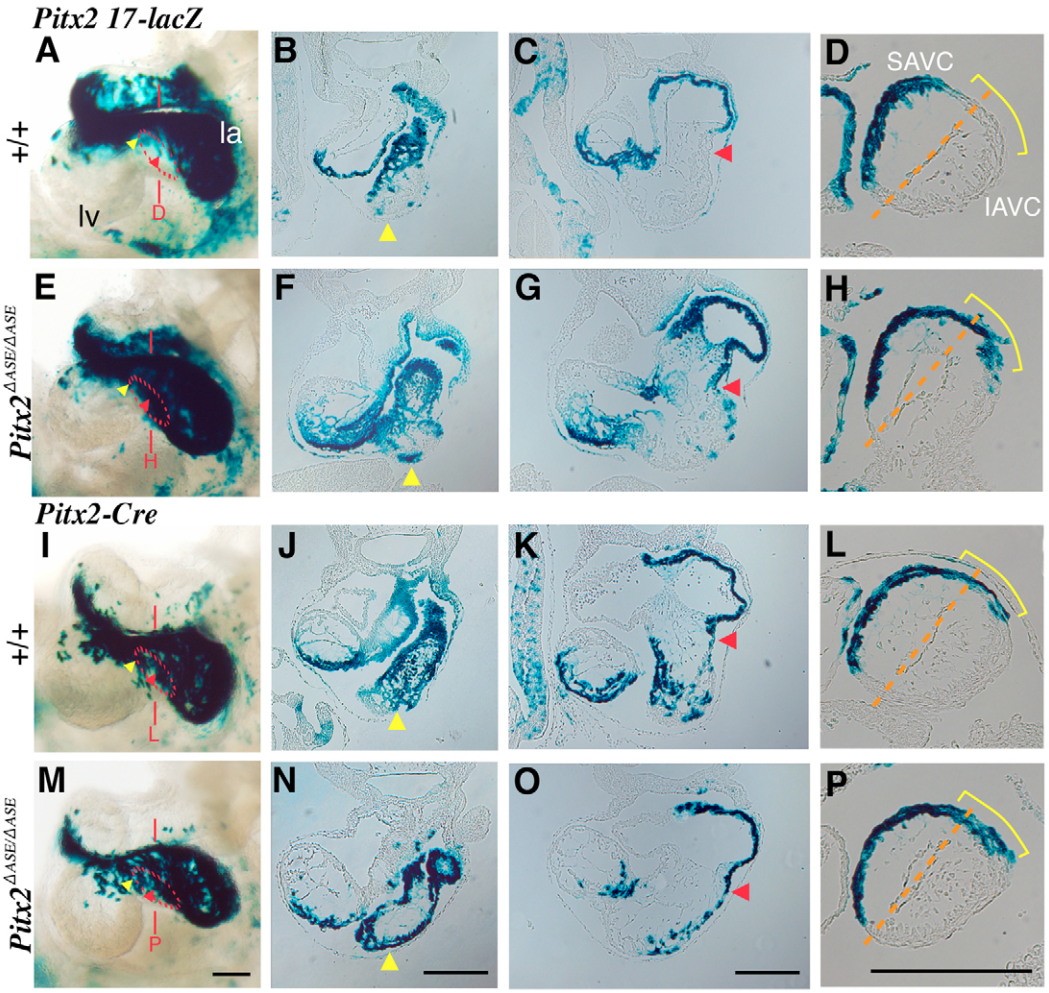


**Fig. 8. Regulation of *Pitx2* expression by negative feedback in the ventricle and AV canal of the heart.** (**A-H**) Expression of the *Pitx2 17-lacZ* transgene in the heart of wild-type (A-D) or $Pitx2^{\Delta ASE/\Delta ASE}$ (E-H) embryos at E9.5. (**I-P**) Expression of the *Pitx2-Cre* transgene in wild-type (I-L) or $Pitx2^{\Delta ASE/\Delta ASE}$ (M-P) embryos at E9.5 as monitored with the *CAG-CAT-lacZ* transgene. A left view of the heart is shown in the left-most panels. Red dotted circles indicate a large portion of the ventricle and AV canal in which X-gal staining is absent in A but present in E, I and M. The transverse sections of the heart at the planes indicated by the yellow and red arrowheads are shown in B,F,J,N and C,G,K,O, respectively. The right-most panels show sagittal sections at the AV canal. Their planes are indicated by the red vertical lines in A,E,I,M. The boundaries between SAVC and IAVC in D,H,L,P are indicated by the orange dotted lines. It should be noted that X-gal staining is apparent only at the myocardium adjacent to the SAVC in D, while it is also apparent in the myocardium adjacent to the IAVC (H,L,P). The domain that is subject to the negative feedback regulation is indicated by the yellow bar in D,H,L,P. Scale bars: 200 μm. la, left atrium; lv, ventricle of the heart.

DEVELOPMENT

**Table 1. The expression data for various constructs in E8.2 and E10.5 mouse embryos**

**E8.2**

| | Asymmetric expression at LPM | Only ectopic expression | No *lacZ* expression |
|---|---|---|---|
| Mouse *Pitx2* | 10 | 3 | 5 |
| Human *Pitx2* | 5 | 0 | 0 |
| Chicken *Pitx2* | 4 | 5 | 1 |
| Frog *Pitx2* | 5 | 0 | 0 |
| Zebrafish *Pitx2* | 4 | 0 | 0 |

**E10.5**

| | Common atrial chamber | Septum transversum | Foregut (lung bud) | Gut dorsal mesentery |
|---|---|---|---|---|
| Mouse *Pitx2* | +++ | ++ | + | ++ |
| | +++ | ++ | ++ | ++ |
| | ++ | – | – | – |
| Human *Pitx2* | + | +++ | +++ | +++ |
| | – | ++ | +++ | +++ |
| | + | – | – | ++ |
| Chicken *Pitx2* | + | + | ++ | ++ |
| | – | + | +++ | – |
| Frog *Pitx2* | +++ | – | – | ++ |
| | + | – | – | ++ |
| Zebrafish *Pitx2* | – | – | – | – |
| | – | – | – | – |

+, asymmetric expression; –, no expression. The relative level of asymmetric expression is indicated by the number of plus symbols. Multiple embryos were sectioned for all constructs, with each row representing an individual embryo.

To address this issue, we generated permanent transgenic mouse lines that express *Pitx2* either transiently or continuously. These mice thus harbor a *Pitx2* transgene driven either by a mutant ASE (NmASE) that lacks the Nkx2-binding site or by the wild-type ASE (Fig. 9A). In the former transgenic mice (line *Tg50*), the transgene was expressed in LPM at E8.2 (Fig. 9D) but not in visceral organs at E9.5 or 10.5 (Fig. 9G,J,M; data not shown), as expected. In the latter transgenic mice (lines *Tg39* and *Tg55*), asymmetric expression of the transgene was apparent in LPM at E8.2 (Fig. 9B,C) and was maintained at E10.5. However, transgene expression

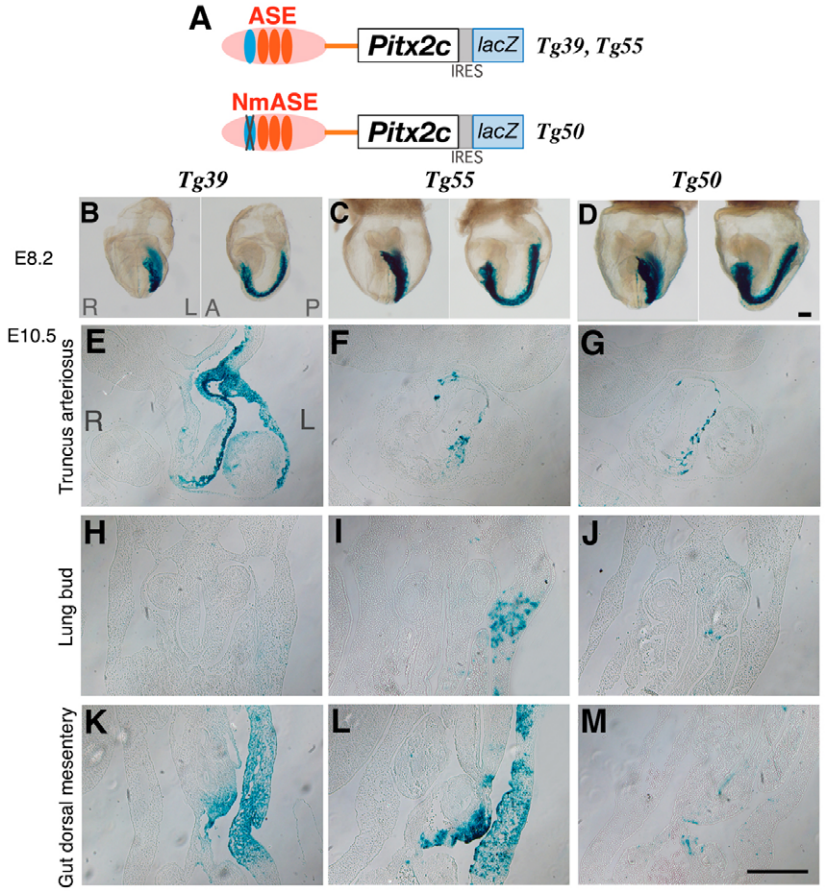


**Fig. 9. Generation of transgenic mice that express *Pitx2c* under the control of the ASE.** (**A**) Construction of two transgenes. Expression of the *Pitx2c* cDNA and *lacZ* is driven either by the wild-type ASE or a mutant version (NmASE) that lacks the Nkx2-binding site. Permanent transgenic mouse lines were established for the former (*Tg39*, *Tg55*) and latter (*Tg50*) transgenes. Red and blue ovals represent Foxh1-binding sites and the Nkx2-binding site, respectively. (**B-M**) Expression of the transgenes in each transgenic line was monitored by X-gal staining. The transgenes in each line (*Tg39*, *Tg55*, *Tg50*) were expressed at similar levels in left LPM at E8.2 (B-D). The transgene of line *Tg39* was asymmetrically expressed in the truncus arteriosus at a high level (E), in gut dorsal mesentery at a low level (K) and not at all in the lung bud (H) at E10.5. Asymmetric expression of the transgene in line *Tg55* was apparent at a high level in gut dorsal mesentery (L), at a low level in the truncus arteriosus (F) and not at all in the lung bud (I). Asymmetric expression of the transgene in line *Tg50* was not detected in any organ examined (G,J,M). Frontal and lateral views of embryos are shown in B-D, and transverse sections are shown in E-M. Scale bars: 200 μm.

DEVELOPMENT

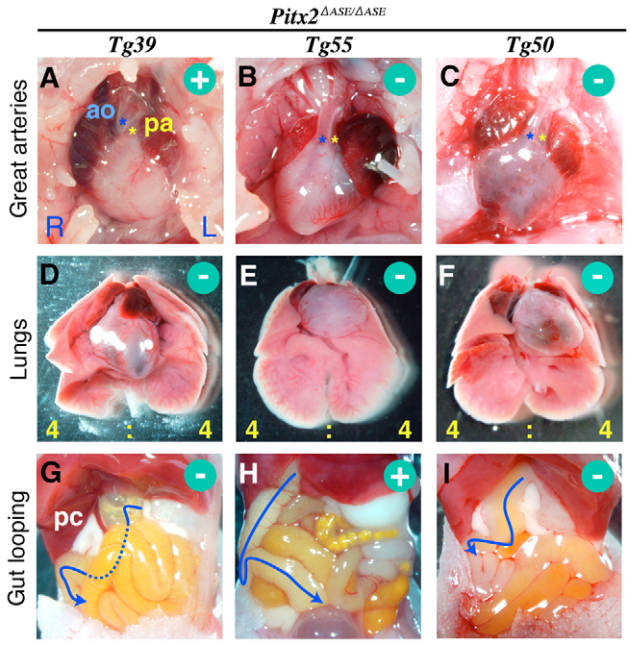


**Fig. 10. Abilities of the *Tg39*, *Tg55* or *Tg50* transgenes to rescue the LR defects of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice.** Phenotypes of *Pitx2*$^{\Delta ASE/\Delta ASE}$ neonates expressing the transgenes of lines *Tg39* (**A**,**D**,**G**), *Tg55* (**B**,**E**,**H**) or *Tg50* (**C**,**F**,**I**) are shown. The transgene of line *Tg39* rescued the great artery defect (A), whereas that of line *Tg55* rescued the abnormal rotation of the gut (H) apparent in *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. The transgene of line Tg50 was unable to rescue any of the defects examined (C,F,I). Right isomerism of the lungs was not rescued by any of the transgenes (D-F); the number of lobes is indicated in yellow. Blue arrows in G-I indicate the location of the duodenum. Encircled + or – signs indicate whether LR defects were rescued or not, respectively. ao, aorta (blue asterisk); pa, pulmonary artery (yellow asterisk); pc, pancreas.

was maintained at E10.5 only in subsets of visceral organs; it was highly expressed in the truncus arteriosus, weakly in the gut dorsal mesentery and not in lung bud in *Tg39* mice (Fig. 9E,H,K), whereas it was highly expressed only in the gut dorsal mesentery in *Tg55* mice (Fig. 9F,I,L). A high level of *Pitx2* expression in some domains may be nonpermissive because we were unable to establish transgenic mouse lines that express the transgene in many visceral organs.

The transgenes in the *Tg50*, *Tg39* and *Tg55* lines were then examined for their ability to rescue the LR defects of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. The transgene of line *Tg39*, which was highly expressed in the truncus arteriosus (Fig. 9E), was able to rescue the morphological defect of the great vessels (11/13 mice, 85%) (Fig. 10A) but not the internal defects of the heart (including ASD, VSD and DORV) or other defects (Fig. 10D,G; data not shown) of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. The transgene of line *Tg55*, which was highly expressed in the gut dorsal mesentery (Fig. 9L), was able to rescue the abnormal rotation of the gut (7/12, 58%) and aberrant positioning of the pancreas (7/12, 58%) (Fig. 10H, data not shown) but not other defects (Fig. 10B,E) of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. Thus, defects were rescued for the organs in which the transgene was highly expressed. Finally, the transgene of line *Tg50* was unable to rescue any of the morphological defects of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice (Fig. 10C,F,I). These results demonstrate that situs-specific morphogenesis requires continuous expression of *Pitx2* on the left side of primordial organs.

## DISCUSSION

### A conserved mechanism for regulation of asymmetric *Pitx2* expression

*Pitx2* is expressed asymmetrically for a long period during organogenesis and executes LR asymmetric morphogenesis in vertebrates (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). Mouse *Pitx2* is regulated by an ASE that contains three Foxh1-binding sequences and an Nkx2-binding sequence, both of which are essential for asymmetric expression (Shiratori et al., 2001). The phenotype of *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice described in the present study now establishes an essential role for the ASE in *Pitx2* regulation. Addition of multiple copies of the Nkx2 binding sequence derived from the ASE of *Pitx2* to the ASE of *Lefty2* transformed the transient action of the latter into a longer lasting one, confirming the essential role of the Nkx2-binding sequence in the ASE of *Pitx2*. We have also now shown that *Pitx2* of all vertebrates (mouse, rat, human, chicken, frog, zebrafish) examined possesses an ASE in the last intron with a similar organization, namely two or three Foxh1-binding sequences and one Nkx2-binding sequence. Asymmetric *Pitx2* expression in vertebrates is thus regulated by a highly conserved enhancer, ASE.

The ASEs of *Pitx2* from each of the various vertebrates examined showed largely similar activities in mouse embryos. The ASE of mouse, human, or chicken *Pitx2* showed activity in the left LPM at E8.2 as well as in many organs, including the common atrial chamber, lung bud, septum transversum and gut dorsal mesentery, at E10.5. By contrast to the ASE of *Pitx2* from other vertebrates, however, that of zebrafish *Pitx2* was active only in the anterior portion of left LPM at E8.2 and was inactive in all organs examined at E10.5. It is thus possible that the Nkx2-binding sequence in the ASE of zebrafish *Pitx2* is recognized by zebrafish Nkx2 but not by mouse Nkx2. Alternatively, the Nkx2-binding sequence of the ASE of zebrafish *Pitx2* alone may not be sufficient for maintenance of *Pitx2* expression.

### Pitx2-dependent and -independent LR organogenesis

The *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice manifested LR defects in many organs. LR asymmetry in some organs, however, remained normal in the *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. This latter finding is not due to residual *Pitx2* expression, which was detected only in the common cardinal vein and vitelline vein. In mutant mice that lack expression of cryptic (Yan et al., 1999) or *Foxh1* (Yamamoto et al., 2003), however, those organs that remain normal in *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice are abnormal. The asymmetric morphogenesis of such organs thus appears to be regulated in a manner independent of Pitx2 but dependent on Nodal signaling.

LR asymmetric morphogenesis is achieved by three mechanisms: (1) directional looping of a tube; (2) differential lobation, as in the lungs or liver; and (3) one-sided regression, as in blood vessels (Hamada et al., 2002). LR asymmetric events regulated in a Pitx2-independent manner include heart looping, embryonic turning and looping of the duodenum-stomach, all of which correspond to the first pattern of morphogenesis. The corresponding organs form initially as a straight tube at the midline that subsequently undergoes looping or turning. Directional looping of the developing heart, for example, may be achieved by physical forces intrinsic to the heart, such as those generated by changes in the arrangement of intracellular actin bundles (Itasaki et al., 1991; Itasaki et al., 1989), changes in myocardial cell shape (Manasek et

al., 1972) or differential rates of cell proliferation. Embryonic turning may also involve LR asymmetric rates of cell proliferation in LPM (Miller and White, 1998). Alternatively, the force for heart looping may be provided externally, such as by the adjacent splanchnopleure (Voronov et al., 2004). Although most situs-specific organogenesis depends on *Pitx2*, the mechanism of Pitx2 action remains unknown. Pitx2-dependent events include the development of asymmetries in lung lobation, blood vessel remodeling and atrial shape, and it remains to be determined how Pitx2 regulates such seemingly different cellular processes.

## Significance of two-step regulation of asymmetric *Pitx2* expression

*Pitx2* is expressed asymmetrically for a long period and is regulated in two steps: initiation by Nodal signaling and maintenance by Nkx2. In the present study, we examined whether the early phase of *Pitx2* expression is sufficient or whether continued expression is necessary for situs-specific organogenesis. Our data obtained from transgenic rescue experiments demonstrate that the persistent expression of *Pitx2* is required. It is possible that Pitx2 regulates various cellular events in organs undergoing LR asymmetric morphogenesis. If so, what is the role of the early phase of *Pitx2* expression in left LPM? The early-phase expression may play a positive role in LR morphogenesis by activating target genes that are essential for morphogenesis in the late phase. Alternatively, the early-phase expression may simply be necessary for the late-phase expression; although Pitx2 might play a role only during the late phase, its expression may need to be initiated at the early phase in response to Nodal signaling. Distinguishing between these possibilities will require the generation of transgenic mice that asymmetrically express *Pitx2* only at the late phase.

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**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/15/3015/DC1

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