Pitx2 determines left–right asymmetry of internal organs in vertebrates

Aimee K. Ryan*†, Bruce Blumberg†‡, Concepción Rodriguez-Esteban†‡, Sayuri Yonei-Tamura†‡, Koji Tamura‡, Tohru Tsukui‡, Jennifer de la Peña‡, Walid Sabbagh‡, Jason Greenwald‡, Senyon Choe‡, Dominic P. Norris§, Elizabeth J. Robertson§, Ronald M. Evans‡‖, Michael G. Rosenfeld* & Juan Carlos Izpisúa Belmonte‡

** Howard Hughes Medical Institute, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0648, USA*
§ Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138, USA
‖ Howard Hughes Medical Institute, ‡ The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037, USA
† These authors contributed equally to this work

The handedness of visceral organs is conserved among vertebrates and is regulated by asymmetric signals relayed by molecules such as Shh, Nodal and activin. The gene *Pitx2* is expressed in the left lateral plate mesoderm and, subsequently, in the left heart and gut of mouse, chick and *Xenopus* embryos. Misexpression of Shh and Nodal induces *Pitx2* expression, whereas inhibition of activin signalling blocks it. Misexpression of *Pitx2* alters the relative position of organs and the direction of body rotation in chick and *Xenopus* embryos. Changes in *Pitx2* expression are evident in mouse mutants with laterality defects. Thus, *Pitx2* seems to serve as a critical downstream transcription target that mediates left–right asymmetry in vertebrates.

The vertebrate body exhibits bilateral symmetry externally whereas the internal organs display significant left–right asymmetry. During organogenesis, the unpaired organs of the chest and abdomen begin development in the midline and then lateralize, with the first morphological markers of left–right asymmetry being the right-sided looping of the developing heart. A second sign of asymmetry is then manifested by the rotation of the body in amniote embryos. Virtually all visceral organs ultimately show left–right asymmetry, either with respect to their location in the body cavity or by morphological differences on one side versus the other. The left–right asymmetries of internal organ placement are invariant within a given species and have been conserved throughout evolution. Normal organ placement is termed *situs solitus*, and the mirror-image arrangement is *situs inversus*. Other defects of *situs* are partial (heterotaxy) or complete (isomerism) loss of asymmetry. Left–right axis malformations in humans are phenotypically variable and genetically heterogeneous[1,2]. Generally, individuals with complete *situs inversus* do not suffer severe clinical consequences, whereas heterotaxia and isomerism are associated with moderate-to-severe physiological complications[3,4].

As the establishment of correct left–right asymmetry is critical for survival, the mechanisms governing initiation and maintenance of these asymmetries should be tightly regulated and evolutionarily conserved. Several models have been proposed to account for these asymmetries (reviewed in refs 5–7). In chick, there is a signalling cascade involving members of the TGF-β superfamily, namely activin-βB and Nodal, the activin receptor RIIA (cAct-RIIA) and Sonic hedgehog (Shh), all of which are asymmetrically expressed with respect to the left–right axis[8,9]. *Activin-βB*, present asymmetrically on the right side of stage 3–5+ embryos[9,10], is thought to induce local expression of *cAct-RIIA*[8,10], which in turn represses the bilaterally symmetrical *Shh* expression in Hensen's node on the right[8,9]. This leads to left-sided expression of *Shh* and induction of *nodal* in the left lateral plate mesoderm[8]. Misexpression of activin or Shh disrupts the normal expression pattern of *nodal* and randomizes heart looping. In *Xenopus*, inappropriate expression of the TGF-β family member Vg-1 inverts *nodal* expression and results in *situs inversus*[11,12]. In contrast to the chicken model, targeted gene deletion of Shh, activin-βB, follistatin or Act-RIIA in mice does not alter the left–right orientation of the heart or of the internal organs, calling into question their role in left–right patterning in the mouse[13–17]. Mice null for *Act-RIIB*, which is not asymmetrically expressed in chick or mouse, exhibit defects in left–right asymmetries, including isomerisms[18], suggesting that Act-RIIB is a critical component of the left–right pathway in mouse.

Of the many molecules that have been implicated in left–right signalling during vertebrate embryogenesis, only Nodal exhibits a clear correlation between its expression in the lateral plate mesoderm and visceral *situs*[19,20]. In *inv/inv* mice, where virtually all animals exhibit *situs inversus*, *nodal* is expressed only in the right lateral plate mesoderm[19,20]. In *iv* mice, where left–right development is randomized, all four possible patterns of *nodal* expression are observed: left, right, bilateral and absent[20] (see also ref. 21). *nodal* expression is bilateral in *Fused toes*[22] and *no turning*[23] mice, which also have randomized left–right asymmetries. Altering the normal *nodal* expression pattern in the left lateral plate mesoderm in *Xenopus* and chick is also associated with changes in left–right development[8,11,24–26]. Thus, Nodal appears to be a conserved factor in the cascade that establishes left–right asymmetry in all vertebrates. The observations that *nodal* expression reliably predicts *situs* and that loss of Act-RIIB function leads to defects in *situs* suggests that these factors function in a common signalling pathway.

Although progress has been made in understanding early events in the determination of left–right asymmetry, much is yet to be learned about how multiple extracellular signals are transduced, propagated and maintained, ultimately leading to visceral asymmetry. Transcription factors are good candidates for mediating these processes. However, relatively little is known of their role in this process, and only three have been implicated in the left–right asymmetry pathway. HNF-3β may have a role because it is transiently asymmetrically expressed in the chick[8] and because *HNF-3β*$^{+/-}$, *nodal*$^{lacZ/+}$ double-heterozygous mice express *lacZ* bilaterally in the lateral plate mesoderm and have defects in the positioning of the viscera and heart, and random embryonic rotation[19]. The zinc-finger gene *Snail-Related* (*cSnR*) which is initially expressed

bilaterally in the presumptive anterior cardiac mesoderm before becoming significantly more intense on the right, is downregulated by ectopic expression of *Shh* on the right, and perturbed by ectopic activin on the left. Antisense experiments designed to disrupt *cSnR* translation reverse heart looping[27]. Finally, the homeodomain factor Nkx2.5 appears to regulate the asymmetric expression of the basic helix–loop–helix (bHLH) factors dHAND and eHAND, which are required for correct heart looping and morphogenesis[28,29].

Here we investigate the role of the bicoid-related homeodomain transcription factor Pitx2 in determining left–right asymmetry in chick, *Xenopus* and mouse. The human homologue of Pitx2, RIEG, was originally described as the gene for Rieger syndrome[30], an autosomal dominant human disorder characterized by ocular anterior chamber anomalies, dental hypoplasia, mild craniofacial dysmorphism and umbilical stump abnormalities, together with occasional defects in cardiac, limb and pituitary development. Our results indicate that Pitx2 may turn on the gene network responsible for the morphological events that result in left–right asymmetries in vertebrates. Whereas umbilical and cardiac phenotypes may suggest a link between Pitx2 and heart and gut development, the lack of alteration in organ *situs* in individuals affected with Rieger syndrome may be due to the presence of the wild-type allele. In chick, *Xenopus* and mouse, *Pitx2* expression is on the left side of the embryo in the lateral plate mesoderm; it then continues to be expressed asymmetrically in several organs that are asymmetric with respect to the left–right axis of the embryo. *Pitx2* expression in the left lateral plate mesoderm is preceded by *Shh* and *nodal*, and we find that *Pitx2* expression can be induced by both Shh and Nodal, suggesting that it is downstream of these signalling molecules. In mutant mice with laterality defects, *Pitx2* expression correlates with changes of visceral *situs*, paralleling the expression of *nodal*. Inhibition of signalling through a dominant-negative activin type-II receptor also alters *Pitx2* expression. Finally, ectopic expression of *Pitx2* in the right lateral plate mesoderm results in isomerism, or in reversed looping of the heart and gut and reversed body rotation in chick and *Xenopus* embryos. Our results indicate that Pitx2 may interpret and subsequently execute the left–right developmental program dictated by upstream signalling molecules and they identify Pitx2 as the first evolutionarily conserved transcription factor in the left–right pathway to control embryonic handedness in vertebrates.

Asymmetric expression during embryogenesis

Chick and *Xenopus Pitx2* (mammalian homologues *RIEG*[30], *Pitx2* (ref. 31), *Potxlx2* (ref. 32), and *Apr-1* (ref. 33)) were isolated by screening chick and *Xenopus* complementary DNA libraries with the murine P-OTX cDNA clone[34,35]. The overall identities for these *Pitx2*

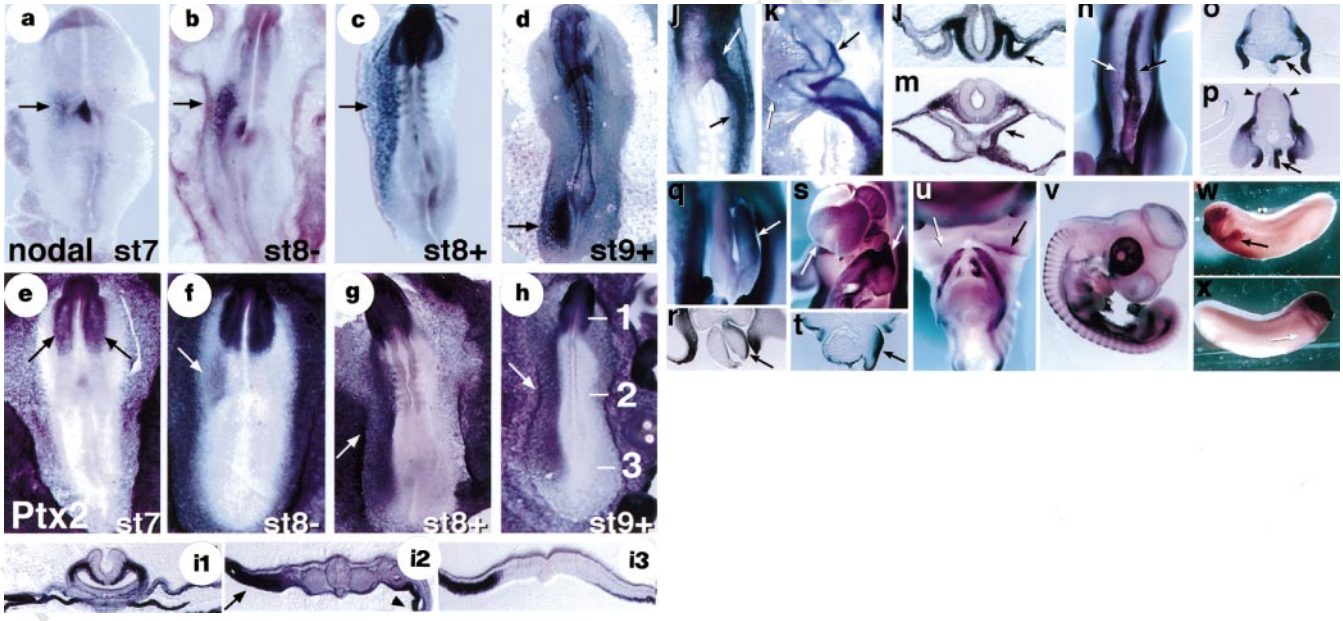

Figure 1 *Pitx2* is expressed asymmetrically in the lateral plate mesoderm, heart and gut. In this and other figures, *Pitx2* is referred to by its old name *Ptx2*. **a**–**i**, Dorsal views; **j**–**x**, ventral views. Where views are dorsal, the left side of the embryo corresponds to the reader's left; for ventral views, the left side of the embryo corresponds to the reader's right. **a**, *Nodal* is expressed in a small medial domain immediately lateral and posterior to the node and in the adjacent region of the left lateral plate mesoderm (arrow). **b**, At stage 8$^-$ *nodal* expression in the left lateral plate mesoderm (arrow) extends posteriorly. **c**, *Nodal* is expressed throughout the left lateral plate mesoderm (arrow) at stage 8$^+$. Expression of *nodal* is also seen in the head mesenchyme. **d**, At stage 9$^+$, a strong region of *nodal* expression is detected in the posterior region of the left lateral plate mesoderm. **e**, Stage-7 embryo, showing the symmetrical expression of *Pitx2* in the head mesenchyme (arrows). **f**, **g**, Dorsal views of stage 8$^-$ to 9$^+$ embryos showing *Pitx2* expression in the head mesenchyme and the left lateral plate mesoderm (arrows). **h**, **i**, Transverse sections (**i**) through stage 9$^+$ embryo shown in **h**: the level at which the sections 1, 2 and 3 were obtained is indicated in **h**. **i**(2), *Pitx2* expression is symmetrically detected in the head mesenchyme; in **i**(2), *Pitx2* expression is detected in both the lateral plate mesoderm and endoderm on the left (arrow) but only in the endoderm on the right (arrowhead). **j**, Stage 10 embryo. *Pitx2* is expressed on the left side of the heart tube (white arrow) and left vitelline vein (black arrow). **k**, Stage 12 embryo. *Pitx2* is expressed on the left (black arrow), but not on the right (white arrow) side of the looping heart tube. **l**, **m**, Transverse section of the embryos shown in **j** and **k**, respectively, showing the expression of *Pitx2* in the left heart tube before looping (arrow in **l**) and in the left side of the epimyocardium (arrow in **m**) during heart looping. **n**, Stage 19 embryo. *Pitx2* expression is present on the left side (white arrow) but not the right side (black arrow) of the developing gut and crop. **o**, **p**, Transverse sections of the same embryo showing asymmetric *Pitx2* expression on the left side of the developing gut (arrows). Bilateral expression is observed in the somites (arrowheads) and at the proximal aspects of the developing hindlimb in **p**. **q**, Stage 21 embryo. *Pitx2* mRNA is detected in the left portion of the caeca (arrow) but not on the right. This is also seen in the transverse section in **r** (arrow). **s**, Ventral view of stage 25 embryo. *Pitx2* is expressed in the heart ventricle (left arrow) and in the gizzard (right arrow). **u**, Stage 22 embryo, showing stronger *Pitx2* expression on the left of the second branchial arch (black arrow). **v**, Stage 24 embryo with *Pitx2* expression in the eye, somites and limb muscle. **w**, **x**, In stage 26 *Xenopus* embryos, *Pitx2* is expressed bilaterally in the eye and cement gland. Expression of *Pitx2* occurs in the left lateral plate mesoderm (**w**, arrow) but not in the right lateral plate mesoderm (**x**, arrow).

homologues are: 98% mouse versus human, 97% chicken versus mouse, 96% chicken versus human, and 89% *Xenopus* versus chicken, mouse or human (see Supplementary Information for amino-acid sequence alignment).

Whole-mount *in situ* hybridization was used to reveal the temporal and spatial expression of *Pitx2* messenger RNA during chick embryogenesis. *Pitx2* mRNA is first detected in the head mesenchyme without apparent left–right asymmetry at stage 7 (Fig. 1e). At stage 8^-, *Pitx2* expression is maintained in the head mesenchyme and appears in a small region of the left lateral plate mesoderm (Fig. 1f). By stage 8^+, *Pitx2* mRNA can be detected along the entire left side of the lateral plate mesoderm (Fig. 1g) and this expression pattern remains relatively unchanged through to stage 10 (Fig. 1h, i). The onset of *Pitx2* expression in the left lateral plate mesoderm appears to be later than that of Nodal, which is also expressed in the left lateral plate mesoderm between stages 7–10 (Fig. 1a–d). At stage 10 *Pitx2* is expressed in the developing left heart tube, and later on in the left heart once it starts to loop (Fig. 1j–m, and data not shown). At stage 22, expression in the second branchial arch is stronger on the left side (Fig. 1u). Asymmetric *Pitx2* expression is also observed in the developing gut, caeca, gizzard and intestine between stages 19 and 25 (Fig. 1n–t). Bilaterally symmetrical expression of *Pitx2* in the somites is first apparent at stage 19 and intensifies as development proceeds (Fig. 1p, v). By stage 22, *Pitx2* is expressed in the mesenchymal cells that migrate into the limb buds and give rise to the limb muscles (Fig. 1v, and data not shown).

The spatial expression of *Pitx2* mRNA in *Xenopus* and mouse was also investigated using whole-mount *in situ* hybridization. In *Xenopus*, asymmetric expression of *Pitx2* is observed first at stage 24, becoming more intense as development proceeds in the left lateral plate mesoderm (Fig. 1w, x). At later stages, and as in the chick, *Pitx2* is expressed on the left half of the heart tube at stage 30 and on the left side of the developing intestine at stage 42 (data not shown). A similar asymmetric pattern in the lateral plate mesoderm is seen in the mouse embryo (see Fig. 4). During heart development, *Pitx2* transcripts are detected in the left half of the linear heart tube (six somite embryo) and later on, at heart looping, in the left side of the ventrical, outflow tract and atrium. During the looping of the gastrointenstinal tract, *Pitx2* is expressed on the left side of the linear gut tube in the six-somite embryo and on the left side of the stomach (E11–E16) and caeca (E14–E16) (data not shown).

Two observations suggest a role for Pitx2 in generating left–right asymmetries. First, the asymmetric expression of *Pitx2* in the left lateral plate mesoderm of chick, *Xenopus* and mouse is preceded by the appearance of *nodal* transcripts in the same region[8,19,20,36]. Second, in contrast to transient expression of *nodal*, *Pitx2* expression is maintained on the left side during organogenesis of the heart and the gastrointestinal tract. These results indicate that *Pitx2* expression may be regulated by signalling molecules previously shown to participate in determining left–right asymmetry, such as Shh and Nodal.

Shh and Nodal induce *Pitx2*

To test this possibility, we ectopically expressed *Shh* and *nodal* in the right lateral plate mesoderm of stage-4–6 chick embryos by infection *in ovo* with a replication-competent avian retrovirus containing either *Shh* or *nodal* cDNAs. To assay the reliability of *in ovo* infection, a replication-competent retrovirus expressing green fluorescent protein (GFP) was injected to the right of the node at stage 4 and the extent of viral infection was visualized at stages 8–12. Although many embryos showed a variable level of infection (about half of the injected embryos were poorly infected), about 25% of the injected blastoderms showed strong GFP expression on the right side of the embryo (data not

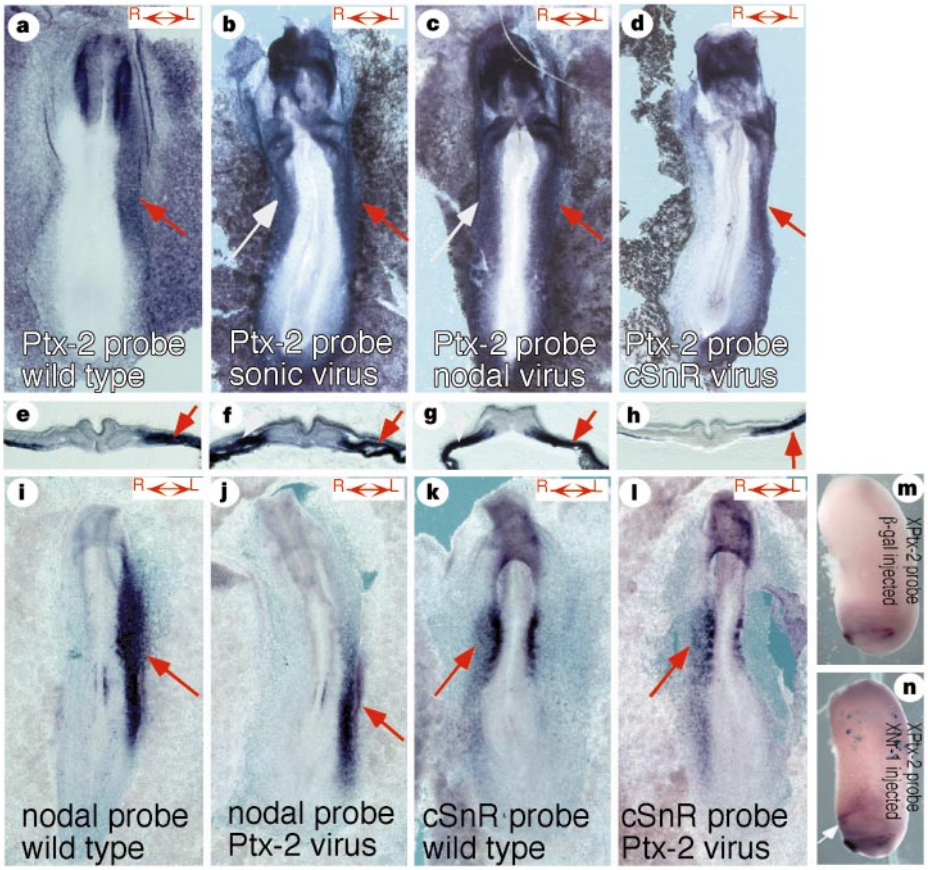

Figure 2 Pitx2 is downstream of Sonic hedgehog and Nodal. Whole-mount *in situ* hybridization of chick (**a**–**l**) and *Xenopus* (**m**, **n**) embryos; ventral views. **a**, Normal *Pitx2* expression in the head mesenchyme and in the left lateral plate mesoderm (arrow). Embryos infected in the right lateral plate mesoderm with RCAS-*Shh* (**b**) or RCAS-*ital-nodal* (**c**) exhibit bilateral *Pitx2* expression in the lateral plate mesoderm (red and white arrows indicate endogenous and ectopic expression respectively). Expression of *Pitx2* is not altered in embryos infected with the zinc-finger protein *Snail-Related* (**d**). **e**–**h**, Transverse sections through the flank of embryos shown in **a**–**d**, respectively. White arrows indicate ectopic expression. (Red arrows indicate endogenous expression.) **i**, Expression of *nodal* in left lateral plate mesoderm of a wild-type embryo. **j**, Expression of *nodal* is unchanged in an embryo infected with RCAS-*Pitx2* (arrow). **k**, Expression of *cSnR* in the right lateral plate mesoderm of an uninfected embryo (arrow). **l**, Expression of *cSnR* is unaffected in embryos infected with RCAS-*Pitx2*. **m**, *Pitx2* is not expressed in right lateral plate mesoderm of a *Xenopus* embryo injected with CMV-βgal. **n**, *Pitx2* expression in the right lateral plate mesoderm of a *Xenopus* embryo injected with *Xnr-1* (white arrow). R⟵⟶L in the right top portion of the panels indicates the orientation of the embryo, R being right and L being left.

shown). In addition, infection *in ovo* does not result in the artefactual changes in *situs* often seen *in vitro* (see below and ref. 8).

Shh and *nodal* injected embryos were fixed between stages 8–12 and expression of *Pitx2* was assessed using whole-mount *in situ* hybridization. Ectopic expression of *Shh* or *nodal* caused bilateral expression of *Pitx2* in the lateral plate mesoderm (Fig. 2a–c, e–g). We also tested the effect of misexpressing the zinc-finger gene *Snail-Related*, *cSnR*, which is required for correct sidedness of heart looping[27]. However, misexpression of *cSnR* in the early chick embryo did not affect the expression of *Pitx2* (Fig. 2d, h).

Similar experiments to determine the ability of Nodal to induce *Pitx2* expression were also done in *Xenopus*. Plasmids expressing *Xnr-1* under the control of the strong cytomegalovirus (CMV) promoter were microinjected into one blastomere of the 4-cell embryo and assayed for *Pitx2* expression at stage 24–26. 86% of the injected embryos that showed exclusive expression of the lineage tracer on the right side exhibited ectopic expression of *Pitx2* on the right side (Fig. 2m, n; see Methods for the number of embryos injected).

To investigate the genetic hierarchy between *Shh*, *nodal* and *Pitx2*, we misexpressed *Pitx2* in the right lateral plate chick mesoderm. There were no detectable changes in *Shh*, *nodal* or *cSnR* expression (Fig. 2i–l and data not shown). Microinjection of *Pitx2* mRNA into *Xenopus* embryos did not affect *Xnr-1* expression either (data not shown). These results suggest that Pitx2 is downstream of Shh and Nodal, and perhaps in a parallel pathway to that of cSnR-1.

Pitx2, organ asymmetry and body rotation

The asymmetric expression of *Pitx2* in the left lateral plate mesoderm, and the observation that its expression can be induced in right lateral plate mesoderm in response to ectopic expression of *Shh* and *nodal* in this region, indicate that Pitx2 may be one of the downstream effectors in the signalling pathway leading to left–right asymmetry. We therefore infected the right lateral plate mesoderm in stage-4–6 chick embryos with an RCAS retroviral vector containing full-length *Pitx2* and scored embryos for changes in heart morphology at stages 11–13. About half of the injected embryos were truncated with respect to their anterior–posterior axis and had a relatively normal heart whose location was shifted towards the head region. The remaining embryos appeared grossly normal and ~55% of these embryos had defects in heart *situs* (Fig. 3). Most of the affected embryos (~70%) had a bilaterally symmetrical heart (isomerism) that was centrally located with respect to the left–right axis (Fig. 3a, b, e), about 25% had reversed heart looping (Fig. 3a, c, d, f), and occasionally double hearts were found (data not shown). Misexpression of GFP did not induce reversal of heart looping or heart isomerisms.

When embryos injected with *Pitx2* at stage 4 were left to develop further, about 12% of the embryos showed a reversal in the direction of embryonic rotation (the embryo turned to the left instead of turning to the right as normal; data not shown). This result suggests that the stronger expression of *Pitx2* on the left side of the body might be relevant at later stages in specifying the direction of body rotation. Similar results regarding the regulation of *Pitx2* by *nodal* and *Shh*, as well as its ability to induce heart isomerism and reversal of heart looping and body rotation, have been obtained independently[37].

Microinjection of *Pitx2* mRNA into a two-cell *Xenopus* embryo showed that, as in the chicken, *Pitx2* overexpression causes alterations in left–right asymmetry (Fig. 3g, h). The predominant phenotype observed was heterotaxy, with the heart being more frequently reversed than the direction of gut coiling, although a few embryos with complete *situs inversus* were obtained (see Fig. 3h, for example).

Altered *Pitx2* expression in *iv* and *inv* mice

Studies of *nodal* expression in *iv* and *inv* mutant mice have shown that the incidence of *situs inversus* is ~50 and 100%, respectively. In contrast to wild-type mice, the expression of *nodal* in *inv/inv* mice is always in the right lateral plate mesoderm[19,20]. In *iv/iv* embryos, the expression of *nodal* is occasionally in the left lateral plate mesoderm, but more often its pattern of expression is altered, being expressed for example only on the right, bilaterally, or not at all in the lateral plate mesoderm[20]. If Pitx2 is an important factor in the left–right pathway and acts downstream of Nodal, its expression in these mouse mutants should parallel the observed patterns of *nodal* expression, which is what we found. In the embryos from an

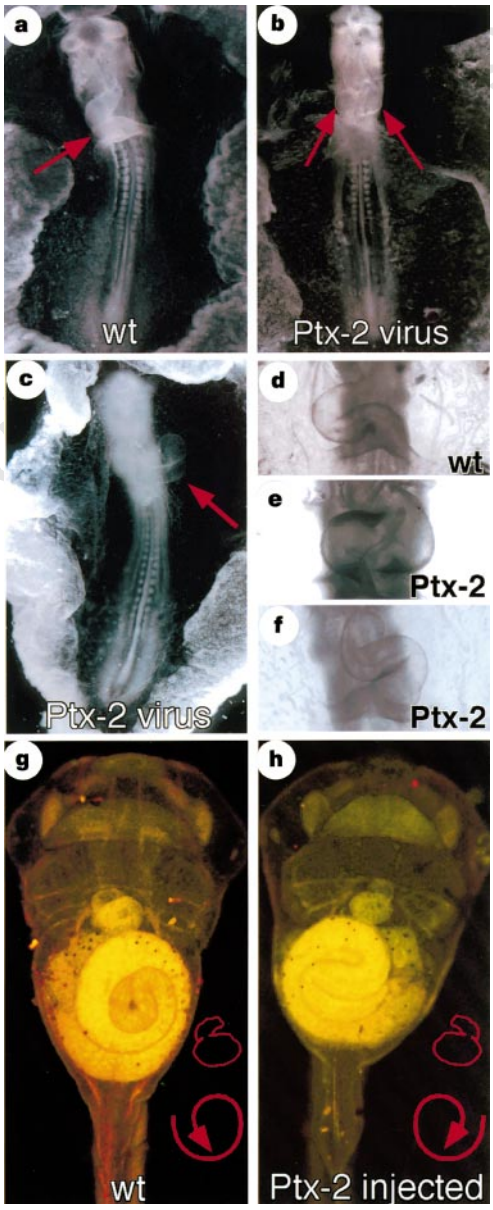

Figure 3 Ectopic expression of *Pitx2* affects left–right asymmetry of the heart and the gut. All embryos are shown from the ventral side. **a**, Wild-type stage 12 chick embryo with normal rightward heart looping (arrow). **b**, Stage 13 chick embryo infected with RCAS-*Pitx2* in the right lateral plate mesoderm at stage 4, showing a bilaterally symmetrical heart. Note the midline location of the bilaterally symmetrical heart with no bias towards the left or right side of the embryo. **c**, Stage 13 chick embryo that was infected with RCAS-*Pitx2* in right lateral plate mesoderm at stage 4 showing leftward heart looping (arrows). **d**, **e**, **f**, Higher magnification at stage 13 of *Pitx2* virus-infected hearts. **d**, Control embryo; heart looping is to the right. **e**, *Pitx2* infected embryo; bilaterally symmetrical heart without a defined looping towards either the left or right side of the embryo. **f**, *Pitx2* infected embryo; heart looping is to the left. **g**, Wild-type stage 45 *Xenopus* embryo with rightward looping heart and a counterclockwise coiled gut. **h**, Stage 45 *Xenopus* embryo that was injected with *Pitx2* at the 4-cell stage, with leftward heart looping and a clockwise coiled gut.

inv/+ × *inv*/+ cross, *Pitx2* was expressed only in the right lateral plate mesoderm in 4/17 mice, which is the expected number of *inv*/*inv* embryos from such a cross (Fig. 4b). In *iv*/*iv* embryos, expression of *Pitx2* in the lateral plate mesoderm was random (Fig. 4c–f): in 20% (3/15) of them, *Pitx2* was expressed in the left lateral plate mesoderm, as in the wild type (Fig. 4a, c); in 20% (3/15), there was no *Pitx2* expression in the lateral plate mesoderm (Fig. 4d); in 20% (3/15), *Pitx2* expression was in the right lateral plate mesoderm (Fig. 4e); and in the remaining 40% (6/15), *Pitx2* was expressed bilaterally (Fig. 4f).

Effect of dominant-negative ActRII on *Pitx2*

In the chick, activin-βB affects left–right asymmetries[8,9]. The action of activin is effected by the asymmetric distribution of targets, presumably activin receptors. In mice, only deletion of activin receptor IIB results in defects in the left–right axis[13,16–18]. Injection of a dominant-negative form of the type II activin receptor in *Xenopus* altered cardiac looping and *Xnr-1* expression when injected on the left side of 16-cell embryos, presumably by interfering with normal Vg-1 signalling[11].

To examine the relation between activin type II receptors, *Pitx2* expression, and visceral *situs*, we used a soluble dominant-negative receptor, ActRII-ECD, which contains the entire extracellular ligand-binding domain of the type II receptor and binds to activin with an affinity comparable to that of the intact receptor[38] (Fig. 5). When present in molar excess, this construct should therefore block activin signalling. Beads coated with ActRII-ECD (0.5 mg ml^{-1}) were implanted into the left or right side of Hensen's node, or immediately above it at stage 4–5 in New culture[10,39]. When beads were implanted away from the node in either the right or left presumptive lateral plate mesoderm, about 30% of the embryos showed reversal of heart looping or bilateral hearts (Fig. 6g). The frequency of reversed heart looping and/or isomerism was higher when beads were placed in the midline (45%). As previously observed, control beads showed a 15% reversal of heart looping[8]. Implanting the dominant-negative activin receptor type II beads had no effect on *Shh* ($n = 24$) expression, regardless of which side they were implanted on (data not shown), although they could induce *nodal* and *Pitx2* expression bilaterally in the lateral plate mesoderm (Fig. 6a–e). In a few embryos, *Pitx2* was not detected in either the left or the right lateral plate mesoderm, and in one embryo, *Pitx2* was expressed only in the right and not in the left lateral plate mesoderm (Fig. 6b). Taken together with the results of *nodal* misexpression and *Pitx2* expression in mutant mice, these results place *Pitx2* downstream of the left–right asymmetry pathway initiated by several members of the TGF-β superfamily.

Conservation of mechanism of L–R asymmetry

As the TGF-β superfamily appears to be important in left–right patterning in several vertebrate species, we propose that these signalling molecules induce a specific transcription factor(s) that is critical for mediating left–right patterning information. We have presented evidence that Pitx2 functions as the transcriptional mediator of left–right *situs*, being required and sufficient to induce the remainder of the downstream program needed to establish morphological asymmetries along this axis. Induction of Pitx2 can be linked to signalling molecules critical for left–right patterning.

In the chick, asymmetric expression of *activin-βB* on the right side of the node between stages 3 and 5$^+$ is the first molecular marker of left–right asymmetry[9]. The sequence of events probably involves activin-βB functioning through cAct-RIIB to activate *cActRIIA* expression on the right side of Hensen's node, leading to down-regulation of *Shh* on the right side of the node. The asymmetric expression of *Shh* on the left side of the node at stage 4 leads to the induction, in the left lateral plate mesoderm, of *nodal*, the second member of the TGF-β superfamily that regulates left–right asymmetry decisions in the chick[8,9]. Shh signalling is both necessary and sufficient for *nodal* induction in the chick[26]. In contrast to the chick model, targeted gene deletions of Shh, activin-βB and the activin receptor IIA in the mouse have no apparent effect on visceral *situs*[13,14,16,17]. Although apparent differences between species may indicate that some components of the molecular machinery responsible for establishing left–right differences are not conserved[26], we suggest that this reflects functional redundancies and that the basic flow of regulatory events is highly conserved among vertebrates.

Nodal shows absolute conservation of expression in all vertebrate species that we have examined. Left-sided expression of *nodal* in the lateral plate mesoderm appears to be a prerequisite for establishing

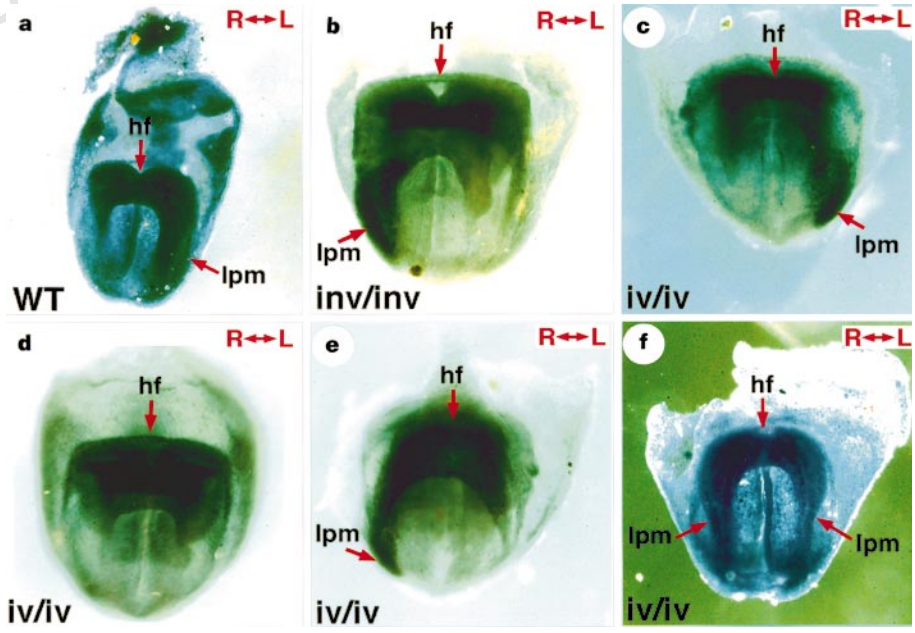

Figure 4 Expression of *Pitx2* is altered in *iv* and *inv* mice. Whole-mount *in situ* hybridization of 8.0 d.p.c. mouse embryos with *Pitx2*. **a**, *Pitx2* is expressed in the head-fold (hf) and left lateral plate mesoderm (lpm) in the wild-type mouse embryo. **b**, In *inv/inv* mice, *Pitx2* expression is observed in the head-fold and right lateral plate mesoderm. **c**–**f**, Four different expression patterns of *Pitx2* in *iv/iv* mice: *Pitx2* in head-fold and left lateral plate mesoderm (**c**); *Pitx2* in head-fold but absent in lateral plate mesoderm (**d**); *Ptx2* in head-fold and in right lateral plate mesoderm (**e**); and, bilateral expression in the lateral plate mesoderm (**f**).

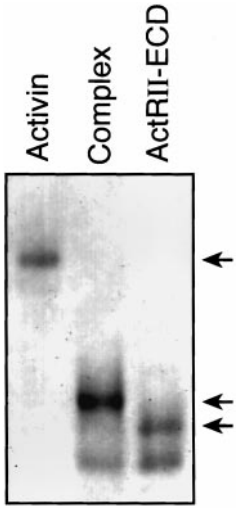

Figure 5 ActRII-ECD binds activin. Non denaturing gel demonstrating the binding of activin and deglycosylated ActRII-ECD. Deglycosylation does not significantly affect the binding affinity. Lanes contain (from left): 1 μg activin, 1 μg activin mixed with 3 μg ActRII-ECD, and 3 μg ActRII-ECD; staining was with Coomassie blue. Arrows indicate protein bands.

the normally invariant pattern of left-right asymmetries. Any variation from this normal *nodal* expression pattern, be it bilateral, absent, or only on the opposite (right) side, has a dramatic effect on the *situs* of the internal organs[8,19,20,26,40]. In mice, two closely related members of the TGF-β family, *Lefty-1* and *Lefty-2*, are expressed in the left ventral midline of the gastrulating embryo and in the left lateral plate mesoderm, respectively[41]. *Lefty-1* and *2* expression in *iv*, *inv*, *Ft* and *no turning* mice parallel *nodal* expression[22,23,41,42]. Ablation of Lefty-1 in the mouse causes bilateral expression of *nodal*[43], suggesting that Lefty-1 signalling participates in the mechanism that normally confines *nodal* expression to the left lateral plate mesoderm.

Our results in the chick using a dominant-negative activin type II receptor agree with the results of targeted gene deletion in the mouse[13,16–18] and from *Xenopus* microinjection[11]. The extracellular domain of the activin type II receptor used as a dominant-negative receptor in our experiments contains the entire ligand-binding domain of the type II activin receptor and can presumably interfere with molecules that normally bind to IIA or IIB type receptors. An explanation of our data from the chick, in which there is bilateral *nodal* expression after applying this dominant-negative ActRII construct, is that Lefty-1 signalling has been blocked in the chick, allowing the spread of *nodal* expression. The defects in heart *situs* in the chick could result from the bilateral expression of *nodal*; interference with Nodal function, or both. Taken together, our results indicate that Nodal, and perhaps Lefty or another similar factor, are key conserved signalling molecules that are required for establishing left–right asymmetry during embryogenesis. We suggest that, although there are apparent differences in the expression of some regulatory genes among model systems, the central mechanism for regulating left–right asymmetry has been widely conserved in vertebrate evolution.

We also suggest that Pitx2 is the central induced effector of the programme mediating left–right patterning. In chick, *Xenopus* and mouse, *Pitx2* expression is first observed on the left side of the embryo in the lateral plate mesoderm. In the chick, *Pitx2* expression in the left lateral plate mesoderm at stage 8 occurs several hours after asymmetric *Shh* expression in the node at stage 4^+ and *nodal* expression in the left lateral plate mesoderm at stage 7. In mice with *situs inversus*, *Pitx2* expression parallels that of *nodal*, and in the chick and frog, misexpression of *nodal* (or *Shh*) induces ectopic *Pitx2* expression. Interference with signalling by TGF-β family members using the dominant-negative activin type II receptor alters *Pitx2* expression and *situs*. In agreement with its predicted function, ectopic expression of *Pitx2* in the right side of the embryo affects left–right asymmetry of the heart and gut and reverses the direction of embryonic turning, resulting in phenotypes similar to those associated with *Shh* and *nodal* misexpression.

Although the different signalling molecules described here are transiently expressed and disappear before morphological asymmetries are visible, *Pitx2* expression is initiated at the onset of organogenesis and is maintained throughout embryogenesis. *Pitx2* is asymmetrically expressed both in the left lateral plate mesoderm, which appears to be the source of the inducing signal directing the morphological movements that give rise to left–right asymmetries, and in the lateral plate mesoderm derivatives that respond to these migration-inducing signals. The left–right instructive role of Pitx2 is not restricted to the heart but is also required for correct *situs* of the gut and body rotation. Thus, to our knowledge, Pitx2 is the first transcription factor demonstrated to regulate left–right asymmetry along the body axis, suggesting that Pitx2 is an

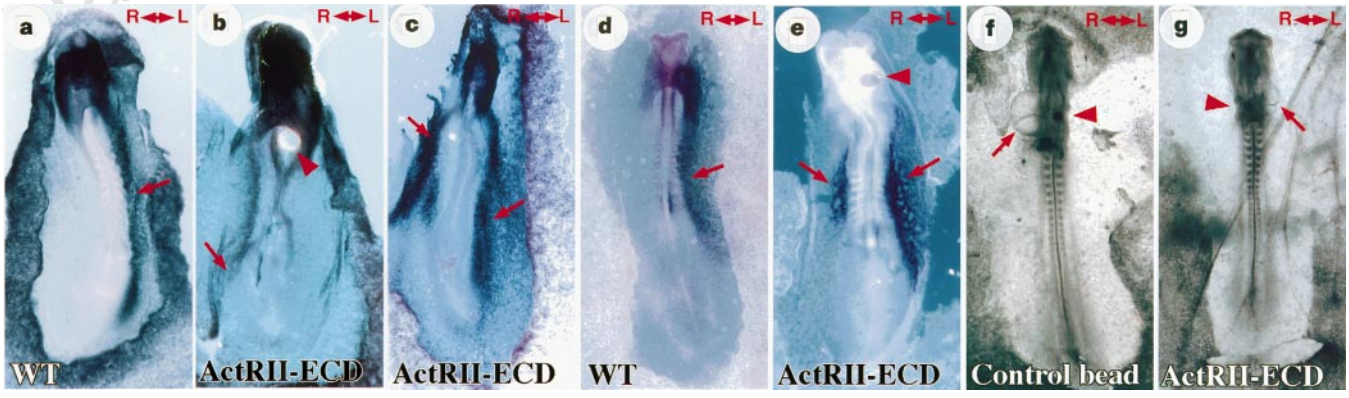

Figure 6 A dominant negative activin receptor alters expression of *nodal* and *Pitx2* and causes reversal of heart looping. All embryos are shown from the ventral side. **a**, Wild-type *Pitx2* expression. **b**, Ectopic *Pitx2* expression on the right lateral plate mesoderm (arrow) of an embryo with ActRII-ECD bead (arrowhead) implanted on the right side at stage 4. Note the absence of *Pitx2* expression on the left side. **c**, *Pitx2* expression in head-folds and bilaterally in lateral plate mesoderm (arrows) of an embryo with ActRII-ECD bead implanted on the right side at stage 4. **d**, Wild-type expression of *nodal* in the left lateral plate mesoderm (arrow). **e**, Embryo in which an ActRII-ECD bead (arrow head) had been implanted on the right side at stage 4, showing ectopic *nodal* expression in the right lateral plate mesoderm (arrow). **f**, Normal rightward looping of the heart (arrow) in an embryo in which a control bead (arrowhead) had been implanted on the right side at stage 4. **g**, Leftward looping of heart (arrow) in an embryo in which a ActRII-ECD bead (arrowhead) had been implanted on the right side at stage 4. R← →L in the right top portion of the panels indicates the orientation of the embryo, R being right and L being left.

evolutionarily conserved downstream effector of the signalling cascade that establishes asymmetries along the entire left–right axis in vertebrates. □

Methods

cDNA cloning and *in situ* hybridization. Stage 22–24 chick limb bud and stage 26 *Xenopus* cDNA libraries were screened at reduced stringency with full-length mouse P-OTX[35]. cDNAs were sequenced on both strands and the sequences deposited in Genbank (AF077092 and AF077767). Whole-mount *in situ* hybridization and sectioning of chick embryos (staged according to ref. 44) were done as described[45]. Antisense probes for *Xpitx2* and c*SnR* spanned the entire ORF, *cPitx2* the homeodomain and C terminus; *Nodal*[8], *Shh*[46] and Xnr-1 (ref. 40) were produced as described[47].

Retroviral infection. RCASBP(A) retrovirus stocks were produced[48] containing full-length cPitx2, Shh or cSnR or mature chick Nodal fused with the BMP-4 proregion[8]. Embryos were infected by right-side blastoderm injection at stages 4–6 (ref. 49).

Construction and microinjection of CDG-Xnr-1 and CDG-XPtx2. The protein-coding regions of *Xnr-1* or *XPitx2* were PCR-amplified from a *Xenopus* gastrula library[50] (*Xnr-1*) or cDNA (*XPitx2*) and cloned into pCDG1 (ref. 51): 100 pg, plus 20 pg of CS2-β-gal lineage tracer, were microinjected into one blastomere of 4-cell albino embryos. Fixation was at stage 24–26, and was followed by β-galactosidase staining[47].

Surviving plasmid-injected embryos (63) were scored according to the location of the lineage tracer: 23 showed exclusive expression on the left and 21/23 (91%) had normal left Pitx2 staining, whereas 2/23 (9%) had bilateral Pitx2 expression. 21/63 showed exclusive expression of the lineage tracer on the right; 3/21 (14%) showed normal left expression of Pitx2, 12/21 (57%) showed bilateral Pitx2 expression and 6/21 (29%) showed reversed right expression of Pitx2. 19/63 could not be scored owing to bilateral or absent lineage tracer expression. All 58 embryos injected with β-gal alone showed normal Pitx2 expression.

Sense mRNA for microinjection was prepared as described[47]. Pitx2 mRNA was injected into pigmented embryos for phenotypic or albino embryos for *in situ* analysis. For phenotypic analysis, embryos were fixed at stage 45, stained with the MF-20 antibody[40] and analysed by light or confocal laser scanning microscopy. *In situ* hybridization was done as described[47].

Protein purification and bead implantation. Chick embryos were grown *in vitro* essentially as described[39] but with modifications[10]. The entire extracellular ligand-binding domain (ActRII-ECD; residues 1–116) of rat activin type II receptor was overexpressed in *Pichia pastoris*[35]. The protein was purified by nickel-affinity chromatography, followed by ion-exchange and size chromatography, and was shown to be monomeric in solution by analytical ultracentrifugation[35]. ActR-ECD was deglycosylated by endoglycosidase H (Glyko) and bound to activin (gift from W. W. Vale) with a dissociation constant of ~10 nM. Affigel blue beads were soaked for 1 h with ActRII-ECD protein (0.5 mg ml^{-1}) and implanted between the hypoblast and epiblast to the right, left or in the midline of stage 4 embryos.

Received 20 May; accepted 9 July 1998.

1. Burn, J. Disturbance of morphological laterality in humans. In *Biological Asymmetry and Handedness* (eds Bock, G. R. & Marsh, J.) 282–299 (Wiley, New York, 1991).
2. Kosaki, K. & Casey, B. Genetics of human left–right axis of malformations. *Sem. Cell Dev. Biol.* **9,** 89–99 (1998).
3. Supp. D. M., Bruickner, M. & Potter, S. S. Handed assymetry in the mouse: Understanding how things go right (or left) by studying how they go wrong. *Sem. Cell Dev. Biol.* **9,** 77–87 (1998).
4. Splitt, M. P., Burn, J. & Goodship, J. Defects in the determination of left–right asymmetry. *J. Med. Genet.* **33,** 498–503 (1996).
5. Wood, W. B. Left–right asymmetry in animal development. *Annu. Rev. Cell Dev. Biol.* **13,** 53–82 (1997).
6. Lander, A., King, T. & Brown, N. A. Left–right development: Mammalian phenotypes and conceptual models. *Sem. Cell Dev. Biol.* **9,** 35–41 (1998).
7. Levin, M. & Mercola, M. The compulsion of chirality: towards understanding of left–right asymmetry. *Genes Dev.* **12,** 763–769 (1998).
8. Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. & Tabin, C. A molecular pathway determining left–right asymmetry in chick embryogenesis. *Cell* **82,** 803–814 (1995).
9. Levin, M. *et al.* Left/right patterning signals and the independent regulation different aspects of *situs* in the chick embryo. *Dev. Biol.* **189,** 57–67 (1997).
10. Stern, C. D. *et al.* Activin and its receptors during gastrulation and the later phases of mesoderm development in the chick embryo. *Dev. Biol.* **172,** 192–205 (1995).
11. Hyatt, B. A., Lohr, J. L. & Yost, H. J. Initiation of vertebrate left–right axis formation by maternal Vg1. *Nature* **384,** 62–65 (1996).
12. Hyatt, B. A. & Yost, H. J. The left–right coordinator: the role of Vg1 in organizing left–right axis formation. *Cell* **93,** 37–46 (1998).
13. Matzuk, M. M., Kumar, T. R. & Bradley, A. Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature* **374,** 356–360 (1995).
14. Chiang, C. *et al.* Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383,** 407–413 (1996).
15. Matzuk, M. M. *et al.* Multiple defects and perinatal death in mice deficient in follistatin. *Nature* **374,** 360–363 (1995).
16. Matzuk, M. M. *et al.* Functional analysis of activins during mammalian development. *Nature* **374,** 354–356 (1995).
17. Vassalli, A., Matzuk, M. M., Gardner, H. A., Lee, K. F. & Jaenisch, R. Activin/inhibin βB subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes Dev.* **8,** 414–427 (1994).
18. Oh, S. P. & Li, E. The signaling pathway mediated by the type IIB activin receptor controls axial patterning and lateral asymemtry in the mouse. *Genes Dev.* **11,** 1812–1826 (1997).
19. Collignon, J., Varlet, I. & Robertson, E. J. Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature* **381,** 155–158 (1996).
20. Lowe, L. A. *et al.* Conserved left–right asymmetry of nodal expression and alterations in murine *situs inversus*. *Nature* **381,** 158–161 (1996).
21. Supp, D. M. *et al.* Mutation of an axonemal dynein affects left-right asymmetry in *inversus viscerum* mice. *Nature* **389,** 963–966 (1997).
22. Heymer, J., Kuehn, M. & Rüther, U. The expression pattern of nodal and Lefty in the mouse mutant Ft suggests a function in the establishment of handedness. *Mech. Dev.* **66,** 5–11 (1997).
23. Melloy, P. G. *et al.* No turning, a mouse mutation causing left–right and axial patterning defects. *Dev. Biol.* **193,** 77–89 (1998).
24. Lohr, J. L., Danos, M. C. & Yost, J. H. Left-right asymmetry of a *nodal*-related gene is regulated by dorsoanterior midline structures during *Xenopus* development. *Development* **124,** 1467–1472 (1997).
25. Levin, M. Left-right asymmetry in vertebrate embryogenesis. *Bioessays* **19,** 287–296 (1997).
26. Pagán-Westphal, S. M. & Tabin, C. J. The transfer of left–right positional information during chick embryogenesis. *Cell* **93,** 25–35 (1998).
27. Isaac, A., Sargent, M. G. & Cooke, J. Control of vertebrate left–right asymmetry by a Snail-related zinc-finger gene. *Science* **275,** 1301–1304 (1997).
28. Srivastava, D., Cserjesi, P. & Olson, E. N. A subclass of bHLH proteins required for cardiac morphogenesis. *Science* **270,** 1995–1999 (1995).
29. Biben, C. & Harvey, R. P. Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene *eHAND* during murine heart development. *Genes Dev.* **11,** 1357–1369 (1997).
30. Semina, E. V. *et al.* Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nature Genet.* **14,** 392–399 (1996).
31. Gage, P. J. & Camper, S. A. Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. *Hum. Mol. Genet.* **6,** 457–464 (1997).
32. Muccielli, M. L., Martinez, S., Pattyn, A., Goridis, C. & Brunet, J. F. Otlx2, and Otx-related homeobox gene expressed in the pituitary gland and in a restricted pattern in the forebrain. *Mol. Cell Neurosci.* **8,** 258–271 (1996).
33. Hirofumi, A. *et al.* Identification and characterization of the ARP1 gene, a target for the human acute leukemia ALL1 gene. *Proc. Natl Acad. Sci. USA* **95,** 4573–4578 (1998).
34. Lamonerie, T. *et al.* Ptx1, a bicoid-related homeobox transcription factor involved in transcription of the pro-opiomelanocortin gene. *Genes Dev.* **10,** 1284–1295 (1996).
35. Szeto, D. P., Ryan, A. K., O'Connell, S. M. & Rosenfeld, M. G. P-OTX: A PIT-1 interaction homeodomain factor expressed during anterior pituitary gland development. *Porc. Natl Acad. Sci. USA* **93,** 7706–7710 (1996).
36. Lustig, K. D. *et al.* A Xenopus nodal-related gene that acts in synergy with noggin to induce complete secondary axis and notochord formation. *Development* **122,** 3275–3282 (1996).
37. Logan, M., Pagán-Westphal, S., Smith, D., Paganesi, L. & Tabin, C. J. The transcription factor of Ptx-2 mediates situs-specific morphogenesis in response to left-right asymmetric signaling. *Cell* (in the press).
38. Donalson, C. J., Vaughan, J. M., Corrigan, A. Z., Fischer, W. H. & Vale, W. W. Characterization of the soluble type II activin receptor extracellular domain. *Biochemistry* (submitted).
39. New, D. A. T. A new technique for the cultivation of the chick embryo *in vitro*. *J. Embryol. Exp. Morphol.* **3,** 326–331 (1955).
40. Sampath, K., Cheng, A. M. S., Frisch, A. & Wright, C. V. E. Functional differences among *Xenopus nodal*-related genes in left–right axis determination. *Development* **124,** 3293–3302 (1997).
41. Meno, C. *et al.* Two closely-related left-right asymmetrically expressed genes, *lefty-1* and *lefty-2*: their distinct expression domains, chromosomal linkage and direct neuralizing activity in *Xenopus* embryos. *Genes to Cells* **2,** 513–524 (1997).
42. Meno, C. *et al.* Left-right asymmetric expression of the TGFβ-family member *lefty* in mouse embryos. *Nature* **381,** 151–155 (1996).
43. Meno, C. *et al.* Left-1 is required for left-right determination as a regulator of Lefty-2 and Nodal. *Cell* (in the press).
44. Hamburger, V. & Hamilton, H. A series of normal stages in the development of the chick embryo. *J. Morph.* **88,** 49–92 (1951).
45. Wilkinson, D. G. in *In Situ Hybridisation* (ed. Wilkinson, D. G.) (Oxford University Press, Oxford, 1993).
46. Yonei, S., Tamura, K., Ohsugi, K. & Ide, H. MRC-5 cells induce the AER prior to the duplicated pattern formation in chick limb bud. *Dev. Biol.* **170,** 542–552 (1995).
47. Blumberg, B. *et al.* An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124,** 373–379 (1997).
48. Vogel, A., Rodriguez, C. & Izpisúa Belmonte, J. C. Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122,** 1737–1750 (1996).
49. Morgan, B. A., Izpisúa Belmonte, J. C., Duboule, D. & Tabin, C. J. Targeted misexpression of Hox-4.6 in the avian limb bud causes apparent homeotic transformations. *Nature* **358,** 236–239 (1992).
50. Cho, K. W. Y., Blumberg, B., Steinbeisser, H. & De Robertis, E. M. The role of the Xenopus homeobox gene goosecoid. Molecular nature of Spemann's organizer. *Cell* **67,** 1111–1120 (1991).
51. Blumberg, B. *et al.* BXR, an embryonic orphan nuclear receptor activated by a novel class of endogenous benzoate metabolites. *Genes Dev.* **12,** 1269–1277 (1998).

Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements. We thank E. Leonardo for her technical skills and dedication; F. H. Gage for access to the confocal laser scanning microscope; D. Peterson for assistance with confocal imaging; C. Tabin for sharing unpublished results; C. V. E. Wright, C. Tabin and L. Erkman and B. Eshelman for reagents and for discussion; J. Magallon and B. Eshelman for technical assistance; and L. Hooks and P. Myer for preparaing the manuscript. S.Y.T. and K.T. were supported by the J.S.P.S. M.G.R. and R.M.E. are investigators of the Howard Hughes Medical Institute; J.C.I.B. is a Pew Scholar. J.G is a HHMI predoctoral fellow. This work was supported by NIH grants to M.G.R., R.M.E. and J.C.I.B., by a G. Harold and Leila Y. Mathers Charitable Foundation grant to R.M.E., S.C., and J.C.I.B.

Correspondence and requests for materials should be addressed to J.C.I.B. (e-mail: belmonte@salk.edu).