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The homeobox gene *Pitx2:* mediator of asymmetric left-right signaling in vertebrate heart and gut looping

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SUMMARY

Left-right asymmetry in vertebrates is controlled by activities emanating from the left lateral plate. How these signals get transmitted to the forming organs is not known. A candidate mediator in mouse, frog and zebrafish embryos is the homeobox gene *Pitx2*. It is asymmetrically expressed in the left lateral plate mesoderm, tubular heart and early gut tube. Localized *Pitx2* expression continues when these organs undergo asymmetric looping morphogenesis. Ectopic expression of *Xnr1* in the right lateral plate induces *Pitx2* transcription in *Xenopus*. Misexpression of *Pitx2* affects situs and morphology of organs. These experiments suggest a role for *Pitx2* in promoting looping of the linear heart and gut.

Key words: Left-right asymmetry, *Pitx2*, Homeobox gene, Mouse, Zebrafish, *Xenopus*

INTRODUCTION

In vertebrates, the organs of the chest and abdomen have a specific non-random asymmetric arrangement with respect to the midline of the body (situs solitus). The apex of the heart points to the left side, the right and left lung display differences in lobation, the liver is on the right side, the stomach and spleen are on the left, and the large intestine curls from right to left (Moore and Persaud, 1993).

Experimental analysis of vertebrate laterality dates back to the 19th century when reversals in asymmetric organ placement (situs inversus) were reported following unilateral warming of chick embryos on the left side (Dareste, 1877). Spemann and his co-workers investigated the origin of body sidedness in amphibians in the early 20th century (reviewed by Wilhelmi, 1921). Three sets of experiments, generation of twinned embryos by ligature, inversion of the middle part of the medullar plate, and unilateral ablations, resulted in defined and predictable laterality defects (Wilhelmi, 1921, and references therein). From these data Wilhelmi concluded that '... the left side of the germ has something that the right side does not have' (Wilhelmi, 1921).

This prediction has been confirmed by the discovery of asymmetrically expressed genes at early stages of embryogenesis, prior to morphological asymmetry, both in the lateral plate mesoderm and at the dorsal midline. Gain- and loss-of-function studies in chick and *Xenopus* have proved the potential of most of these factors to influence laterality. The earliest asymmetric gene activities are found in the chick around the node at the anterior of the primitive streak (activin βB, Levin et al., 1997; *cAct*-RIIa, *HNF3β*, *shh*, *nodal*, Levin et al., 1995). Later, spatially restricted asymmetric gene expression is found in the right (*cSnR*; Isaac et al., 1997) and left (*nodal*; Collignon et al., 1996; Lowe et al., 1996; *lefty*; Meno et al., 1996) lateral plate mesoderm (LPM) in chick, mouse and *Xenopus*.

Recent work in *Xenopus* suggests that once bilateral symmetry is broken by an as yet unidentified activity, a left coordinator transmits an instructive signal to the midline. In the frog, processed Vg1 protein can mimic the function of this coordinator (Hyatt and Yost, 1998). In the chick, it is not known what leads to right-sided expression of activin βB, but this in turn establishes asymmetric *shh* expression in the node. Recent work suggests that left-sided *shh* acts through an additional unidentified downstream signal to induce the TGFβ signaling molecule *nodal* in the left LPM (Pagan-Westphal and Tabin, 1998).

As the embryonic heart and gut undergo asymmetric looping events, transcription factors (*eHAND*, and *dHAND*, Srivastava et al., 1995) as well as components of the extracellular matrix (flectin, Tsuda et al., 1996) and the cytoskeleton (actin, desmin and cytokeratins; Itasaki et al., 1989; Schaart et al., 1989; Price et al., 1996) undergo temporally and spatially restricted activity. Experimental manipulations such as partial loss-of-

function using antisense approaches (*eHAND*, *dHAND*; Srivastava et al., 1995) and interference with the cytoskeleton show an involvement of these factors in the biomechanics of looping (Itasaki et al., 1991).

In contrast, little is known about how the transient LPM signals influence laterality of the developing heart and gut (King and Brown, 1997). In one hypothesis a mediator would be activated by the signaling cascade in the LPM and continue to be expressed in the heart and gut proper. Here we present evidence that the vertebrate homeobox transcription factor *Pitx2* can execute such a function. (1) In mouse, frog and zebrafish embryos *Pitx2* is expressed in the left LPM. (2) In mouse and frog embryos *Pitx2* is also expressed in the tubular heart and gut and continues when these organs undergo looping morphogenesis. (3) In the mouse *iv* mutant *Pitx2* expression is randomized, in much the same way as the expression of *nodal*. (4) Ectopic expression of activin and of the frog nodal homolog *Xnr1*on the right side of *Xenopus* embryos and in animal cap explants leads to the ectopic activation of *Pitx2*, indicating that *Pitx2* might be a target of *nodal* signaling. (5) Misexpression of *Pitx2* in *Xenopus* embryos results in situs defects and altered morphology of heart and gut. Our data suggest that *Pitx2* plays a role at the interface of lateral plate signaling and heart and gut morphogenesis.

MATERIALS AND METHODS

Isolation of mouse, *Xenopus* and zebrafish *Pitx2* cDNA clones

Pitx2-specific sequences were cloned following RT-PCR from P19 cells differentiated for 2 days in 1% DMSO with degenerate primers specific for the *goosecoid* homeodomain (5′AA(A/G)(A/C)GI-(A/C)GICA(C/T)(A/C)GIACIAT(A/C/T)TT(C/T)AC and 5′GCIC-(G/T)IC(G/T)(A/G)TT(C/T)TT(A/G)AACCAIAC). 50 ng cDNA were used for PCR amplification (35 cycles, 60°C/30 seconds, 72°C/90 seconds, 94°C/30 seconds). Among 59 sequenced clones, 57 were *goosecoid* homeobox sequences, 1 specific for *Pitx-1* and 1 for *Pitx2*. Full length cDNA clones were obtained by screening a λZAPII cDNA library made from the same RNA used for the degenerate PCR, following standard procedures (Sambrook et al., 1989).

Xenopus Pitx2 cDNAs were cloned by screening a neurula cDNA library (stage 18, Stratagene) with the entire coding region of mouse *Pitx2* under reduced stringency conditions (hybridization in Quickhyb, Stratagene, at 55°C; final wash 1× SSC/1% SDS at 60°C). Following two rounds of rescreening 36 clones were recovered, 16 of which were sequenced (accession number AJ005786).

A 641 bp fragment of zebrafish *Pitx2* cDNA was cloned by RT-PCR from polyadenylated RNA isolated from 15- to 20-somite embryos using degenerate primers.

In situ hybridization and histological analysis

Whole-mount in situ hybridization protocols followed standard procedures. Analysis of *Pitx2* mRNA expression in thoracic and abdominal organs of E12.5 to E16.5 mouse embryos was performed by whole-mount in situ hybridization of isolated hearts and viscera which were dissected in methanol following standard fixation of embryos in 4% paraformaldehyde.

The following probes were used: mouse *Pitx2* (1.7 kb *Xho*I-*Eco*RI fragment corresponding to entire coding sequence plus 5′ and 3′ UTR), *Xenopus Pitx2* (456 bp *Pst*I fragment, corresponding to nucleotides 316-796 of the sequence submitted to the database, accession number AJ005787); *Xenopus* cardiac troponin I (927 bp *Not*I-*Eco*RI fragment; Drysdale et al., 1994); *Xnr1* (1515 bp *Eco*RI-*Xho*I fragment; Jones et al., 1995), zebrafish *Pitx2* (entire length of cloned fragment).

Histological analysis of whole-mount in situ hybridized embryos was performed following embedding of specimen in paraffin and cutting 12.5 μm sections.

Animal cap assays and semi-quantitative RT-PCR

Embryos were injected with synthetic RNA (*goosecoid* 250 pg; activin 200 pg; *Xnr1* 100 pg; eFGF 20 pg; BMP-4 200 pg) at the 4-8 cell stage into animal blastomeres and grown until stage 8.5. At this stage the animal cap region was excised using eyebrow knives. The explants were cultured in 0.5× MBSH until control embryos reached stage 10.5-11 (gastrula). Total RNA was extracted using a commercial kit (Tristar, AGS) according to the manufacturers instructions. cDNA was synthesized by reverse transcription. Radioactive PCR was performed as described by Ding et al. (1998), using the following primers and conditions: *XPitx2* forward primer GCTCTGGGGAGTGTAAGTCAAG, reverse TTGTTGTACGAG-TAACTGGGGTAC, 29 cycles at 30 seconds/95°C, 30 seconds/57°C, 30 seconds/72°C; EF1α forward CAGATTGGTGCTGGATATGC, reverse ACTGCCTTGATGACTCCTAG, 24 cycles at 30 seconds/95°C, 30 seconds/57°C, 30 seconds/72°C; *Xnr1* forward AGTCAAGTCCTCTGCCAACC, reverse TCAAAACAACCTCA-TCTCCC, 26 cycles at 30 seconds/95°C, 30 seconds/53°C, 30 seconds/72°C; *Xbra* forward CACAGTTCATAGCAGTGACCG, reverse TTCTGTGAGTGTACGGACTGG, 26 cycles at 30 seconds/95°C, 30 seconds/57°C, 30 seconds/72°C; *Xvent1* forward ATCTGACTCTTCAGTTTCATCCGTC, reverse CCAGCGCCGG-CTGAGAACGGCATTC, 26 cycles at 30 seconds/95°C, 30 seconds/55°C, 30 seconds/72°C. *XPitx2* and EF1α amplifications were performed simultaneously in the same tubes.

Injections into *Xenopus* embryos

The coding region of *Xenopus Pitx2* was amplified by PCR and cloned into the vector CS2 (Rupp et al., 1994). Synthetic RNA was prepared using the Ambion message Machine kit. Injections were performed at the 8-cell stage into dorsal blastomeres as specified in the main text. In most experiments a CMV-GFP or a CMV-lacZ construct were co-injected as a lineage tracer. Distribution of green fluorescence was visualized under a Zeiss UV stereo microscope at stage 30-40, and only embryos that were targeted correctly were further cultured. At the end of culture, embryos were processed for immunohistochemistry with the anti-myosin antibody MF-20 and analyzed for situs defects.

RESULTS

Isolation of *Pitx2* genes in mouse, *Xenopus* and zebrafish

Pitx2 cDNA clones were isolated from mouse, *Xenopus* and zebrafish (see Materials and Methods). *Pitx2* was formerly known as RIEG (Semina et al., 1996), *Otlx2* (Mucchielli et al., 1996), *Brx1a* (Kitamura et al., 1997), and *Ptx2* (Gage and Camper, 1997). *Pitx2* belongs to the *bicoid* group of *paired* type homeobox genes which are characterized by a lysine in position 50 of the homeodomain (Hanes et al., 1989). Fig. 1 shows an alignment of the amino acid sequences of the homeo domains of *Pitx2*, which are identical in all species analyzed thus far, with the sequences of mouse *Pitx1* (Lamonerie et al., 1996), *Drosophila Pitx* (*D-Pitx1*, Vorbrüggen et al., 1997) and mouse *goosecoid* (Blum et al., 1992), which is the most closely related homeo domain outside the *Pitx* family. *D-Pitx1* and mouse *Pitx2* resemble each other in a number of ways. First, the homeo domains are

Fig. 1. The *Pitx* family of homeobox genes. Alignment of amino acid sequences of the homeo domains of vertebrate *Pitx2* (identical in human, mouse, chick, zebrafish, and *Xenopus*) with *Drosophila Pitx* (Dpitx), mouse *Pitx1* (mPtx1) and mouse *goosecoid* (mgsc). Only positions that differ from *Pitx2* are shown. The lysine residue characteristic of the *bicoid* group of *paired* type homeoboxes is shown in bold face. Note that the homeo domain of *Drosophila Pitx* displays higher homology to vertebrate *Pitx2* than to *Pitx1*.

closely related (Fig. 1). Second, *D-Pitx1* and mouse *Pitx2* are expressed in a restricted manner in the gut (see below). Third, metameric expression of *D-Pitx1* in the ventral somatic muscles and the CNS is reminiscent of the metameric expression of mouse and *Xenopus Pitx2* in the myotome (data not shown) and brain (Mucchielli et al., 1996). We propose that *Pitx2* is the vertebrate homolog of *D-Pitx1*.

Expression of *Pitx2* during gastrulation, in the left LPM and on the left side of the tubular heart and gut

Pitx2 mRNA expression in the LPM, heart and gut was analyzed in mouse, frog and zebrafish embryos from midgastrula through mid-embryogenesis using whole-mount in situ hybridization. Other sites of expression of the gene such as the head have been published previously (Semina et al., 1996; Mucchielli et al., 1996).

In the mouse the earliest expression was detected in the E7.0 embryo in the head mesenchyme (Fig. 2A). Before embryonic turning asymmetric expression of *Pitx2* mRNA was found in the E8.0 mouse embryo (6 somites) throughout the left LPM and on the left side of the just fused primitive heart tube (Fig. 2B). When the LPM splits into splanchnopleura (inner layer) and somatopleura (outer layer) and the coelom forms, the splanchnopleura is in contact with the endoderm of the primitive gut. Concomitant with embryonic turning the gut tube closes ventrally and the splanchnopleura wraps around the epithelial lining of the gut tube proper. *Pitx2* expression at E9.0 was confined to the left splanchnopleura (Fig. 2C,D). The somatopleura showed *Pitx2* staining on both sides, however signals were stronger on the left side (Fig. 2D).

Localized *Pitx2* mRNA expression in *Xenopus* was first detected at late gastrula/early neurula stage (stage 12; Fig. 2E). The sickle-shaped expression domain marked the anlage of the cement gland from stage 12-18 (Fig. 2E-G). The cement gland remained positive throughout the period monitored, up to stage 45 (Figs 2H, 3A,F). At stage 18 additional mRNA localization was obvious in two triangular patches of head neural crest (Fig. 2G). Asymmetric expression in the left LPM started to be visible at stage 25 (not shown), and was clearly seen from stage 26-35 (Figs 2H, 3A,E; and data not shown). When the endocardial heart tubes fuse to form the linear heart at stage 30, *Pitx2*-specific signals could be detected in a dorsal aspect of the left LPM continuing into the heart region (Fig. 3A,C-E). Staining was clearly confined to the myocardium on the left side at the levels of both the fused (Fig. 3C) and still unfused (Fig. 3D) endocardial tubes. The heart-specific marker cardiac troponin I (cTnI), in contrast, was expressed in the entire myocardium on both sides but did not extend into the more posterior LPM (Fig. 3A,B).

In the zebrafish embryo localized *Pitx2* expression was obvious at 90% epiboly (9 hours) in the polster which is formed by the anterior neural plate at the boundary to the neuroectoderm (Fig. 2I). This expression is consolidated over the next few hours when the derivative of the polster demarcate the boundary of the anterior neural plate (Fig. 2J,K). As in mouse and *Xenopus* asymmetric localization of *Pitx2* mRNA was detected in the left LPM. An example of an embryo at 23 hours is shown in Fig. 2L.

Pitx2 expression during heart and gut looping

Left-sided *Pitx2* expression in the heart continued as it underwent looping morphogenesis at E9.5 (Fig. 4A,D-F). The strongest signal was observed in the common atrium located on the left side of the ventral midline (Fig. 4D,E). Expression in the ventricle and outflow tract was confined to left aspects as well, but was less pronounced (Fig. 4E,F).

The linear gastrointestinal tube of mouse embryos undergoes looping and rotation morphogenesis mainly at two sites, within the midgut, i.e. centering around the junction between small intestine and large intestine and within the caudal part of the foregut, i.e. the future stomach (Fig. 4B,C). Concomitant with gut looping *Pitx2* expression became restricted to these sites. Localized expression in the midgut was obvious in an embryo of 44-48 somites (E11.0) at the apex of the turning midgut loop (Fig. 4G). At E13.5 expression was seen in the caecum (not shown), which remained positive at E16.5 (Fig. 4I). Expression of *Pitx2* in the rotating stomach was observed from E11.0 to E16.5 on the left side, i.e. in the greater curvature (Fig. 4H,I, and data not shown).

Our analysis of *Pitx2* expression during looping stages of the gastrointestinal tract of *Xenopus* embryos was focused on the coiling process of the intestine. The first sign of gut looping is marked by a ventrolateral bulge which forms on the left side of the embryo at about stage 41 and gets drawn out into a first loop at stage 43 (Nieuwkoop and Faber, 1967; Chalmers and Slack, 1998). *Pitx2*-specific mRNA was localized to the left side of this first loop (Fig. 3F). At stage 45 the looping gut has build up a spiral. *Pitx2* expression was detected along the outside of this spiral (Fig. 3G). Staining thus remained confined to the left side of the coiling gut. This can be clearly seen in the diagrammatic representation of the stage 45 embryo and the schematic drawing of the gut coil shown in Fig. 3H, in which *Pitx2* expression is marked in red. As shown for mouse embryos in Fig. 4 localized *Pitx2* expression in the turning heart and stomach was observed in *Xenopus*.

Randomized expression of *Pitx2* in the mouse *iv* mutant

Based on its asymmetric expression we hypothesized a role for *Pitx2* in the process of laterality determination. As an entry point to the analysis of *Pitx2* function we investigated its expression pattern in the mouse *iv* mutant. This strain is characterized by random generation of laterality (Hummel and Chapman, 1959). Approximately half of homozygous *iv* animals display situs solitus, whereas the other half show situs inversus.

Homozygous *iv/iv* mutant embryos ranging in age from 4 to 5 somites up to 9 somites were analyzed by whole-mount in

situ hybridization. All embryos showed the expected *Pitx2* expression domain in the head, and the majority also had expression in the LPM. Four types of *Pitx2* patterns were found: expression in the left LPM (Fig. 5A), on the right side (Fig. 5B), bilaterally (Fig. 5C) or no expression in the LPM at all (Fig. 5D). This randomized pattern suggests a role for *Pitx2* in the establishment of laterality and correlates well with the results seen for nodal in *iv* mutant embryos at the 6- to 8-somite stage (Lowe et al., 1996). In Table 1, *Pitx2* expression data in *iv* mice is compiled along with that previously determined for *nodal*. The correlation suggests the potential for an interaction between *Pitx2* and *nodal* in the determination of laterality.

Table 1. *Pitx2* and *nodal* expression versus sidedness

	Expression patterns in the lateral plate mesoderm			
Gene	Left	Right	Both	None
nodal (29)	7 (24%)	6 (21%)	10 (34%)	6 (21%)
Pitx2 (32)	9 (28%)	8 (25%)	10 (31%)	5 (16%)

Compilation of expression patterns of *Pitx2* and *nodal* in *iv/iv* mutant embryos at 5-9 somites analyzed by whole-mount in situ hybridization.

Induction of *Pitx2* by *nodal* and activin

In *Xenopus* left-sided expression of *Xnr1* commences earlier (stage 19/20, not shown) than *Pitx2* (stage 26), but overlaps later with *Pitx2* in the left LPM (not shown). To assess the effect of ectopic *Xnr1* expression on *Pitx2* expression in both the left and right LPM, we injected *Xnr1* mRNA bilaterally into left and right lateral blastomeres of the 8-cell embryo. Injected embryos were analyzed for *Pitx2* expression at stage 29. Because *Xnr1* injection can lead to secondary axis formation (Sampath et al., 1997), and alterations of dorsoanterior or midline development affect laterality (Danos and Yost, 1996; Lohr et al., 1997; Nascone and Mercola, 1997; Hyatt and Yost, 1998) we injected low amounts of RNA (50 pg per embryo), and scored only embryos with an apparently undisturbed primary axis. While the left-sided expression of *Pitx2* was unaffected by *Xnr1* we observed bilateral expression in 85% of injected embryos (n=26; Fig. 6B,B′). Control injected (Fig. 6A,A′) and uninjected embryos never showed bilateral *Pitx2* expression.

Recently, another TFGβ-like signaling molecule, *activin*,

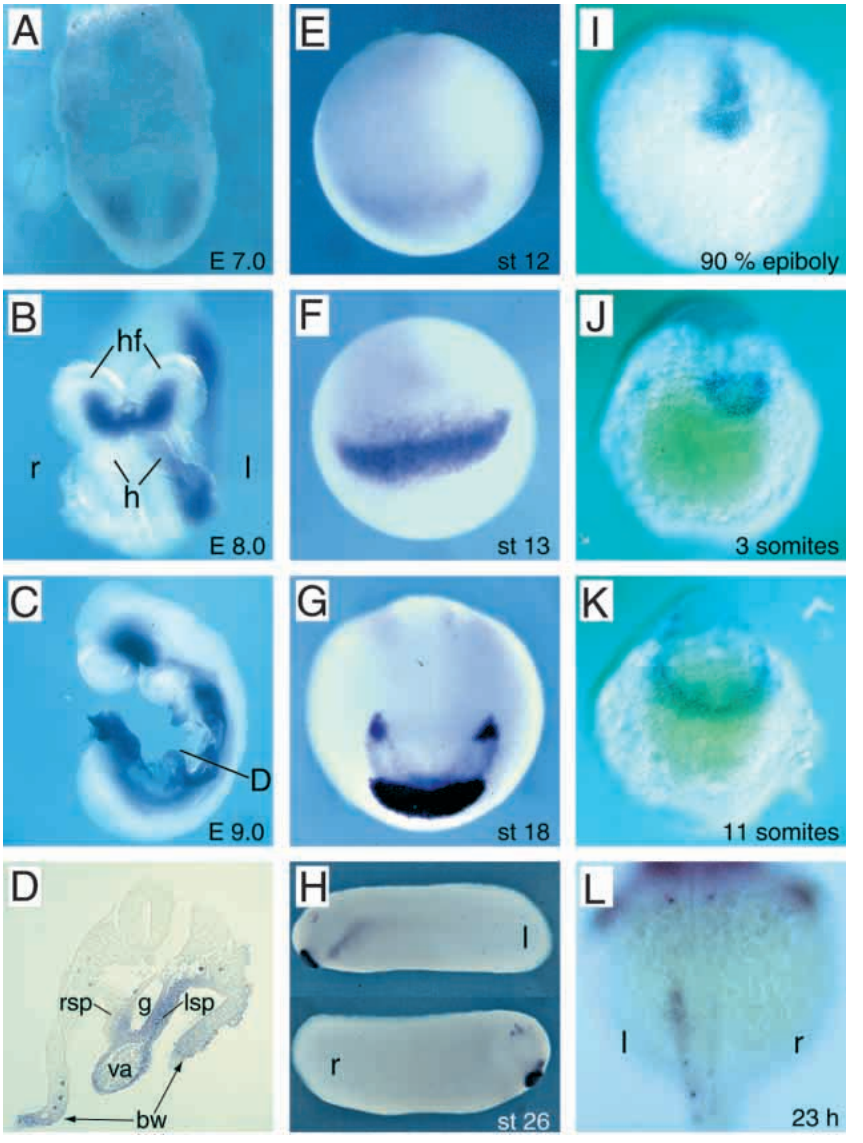


Fig. 2. Left-sided *Pitx2* expression is conserved between mouse, *Xenopus* and zebrafish. (A-D) *Pitx2* expression in the mouse from E7.0 to E9.0. (A) Earliest localized expression of *Pitx2* is seen in two patches in the prospective head mesenchyme. Anterior view of an E7.0 embryo. (B) *Pitx2* is asymmetrically expressed in the left LPM and in the left myocardium of the linear heart tube. Ventral view of a E8.0 embryo (6 somites). hf, headfolds; h, heart; l, left; r, right. (C,D) Left-sided expression of *Pitx2* in the tubular gut at E9.0. Plane of section in D is indicated by the line in C. bw, body wall; g, gut; lsp, left splanchnopleura; rsp, right splanchnopleura; va, vitelline artery. (E-H) *Pitx2* expression in *Xenopus* from stage 12 to stage 26. (E-G) From stage 12 to 18 sickle-shaped expression is seen in the cement gland anlage (E-G) and in two patches of head mesenchyme (G). (H) At stage 26 strong expression persists in the cement gland. Asymmetric expression is seen in the left (top) but not in the right (bottom) lateral plate mesoderm. l, left; r, right. (I-L) *Pitx2* expression in the zebrafish embryo from 90% epiboly to 23 hour in the polster (I-K) and left LPM (L). (I) Animal pole view of an embryo at 90% epiboly (9 hours). (J) Animal pole view of a 3 somite embryo (10 hours). (K) Animal pole view of a 11 somite embryo (14.5 hours). (L) Dorsal view of a 23 hour embryo. Note that the staining is confined to the left LPM. l, left; r, right.

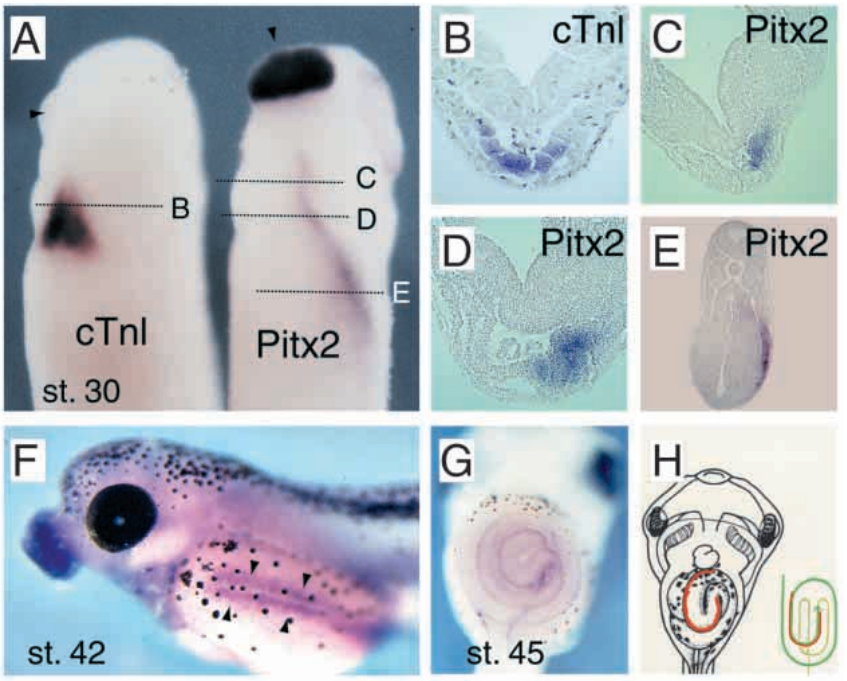
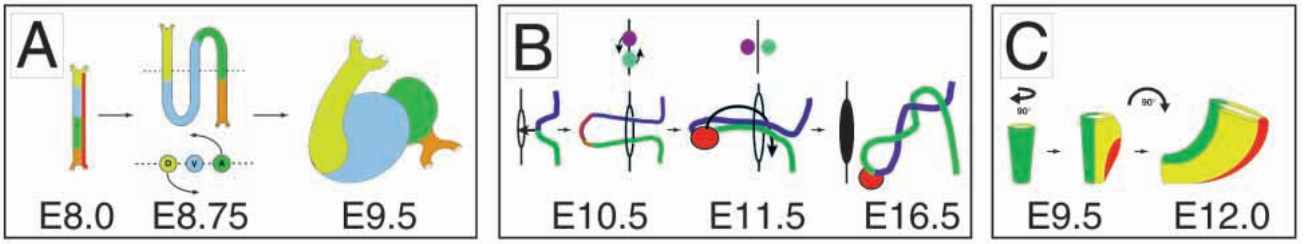


Fig. 3. *Pitx2* expression in the *Xenopus* heart and gut. (A) At stage 30 left-sided *Pitx2* expression (right embryo) extends into the heart region. Staining of an embryo with the myocardial marker cardiac troponin I (cTnI; left) is shown for comparison. Approximate planes of sections in B-E are indicated by transverse lines. (B-E) Histological sections of the embryos shown in (A). Note that *Pitx2* expression is restricted to the left myocardium (C,D) and LPM (E). (F,G) Left-sided *Pitx2* expression in the looping gut at stage 42 (arrowheads in F), and along the outside of the gut spiral at stage 45 (G). (H) Diagram of a stage 45 embryo and a schematic drawing of gut looping (Niewkoop and Faber, 1967). *Pitx2* mRNA expression is indicated in red.



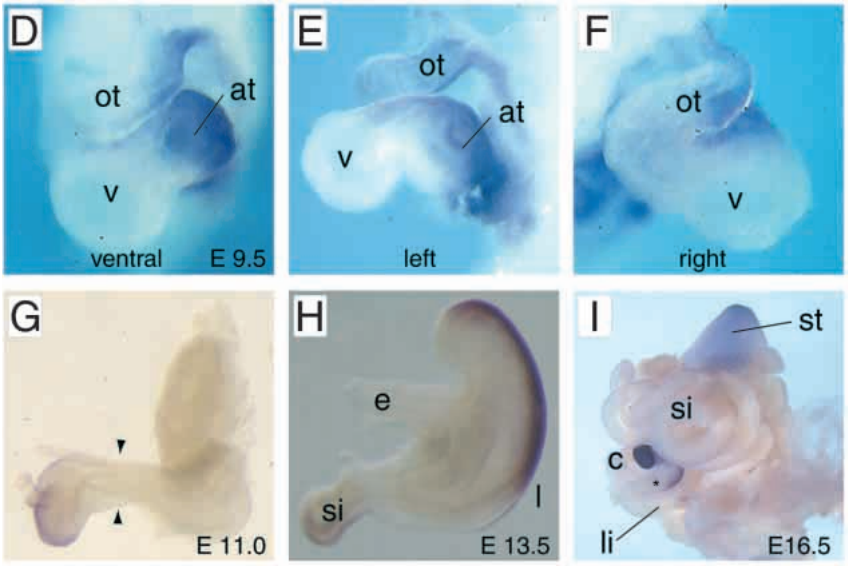


Fig. 4. *Pitx2* expression during heart and gut looping in the mouse. (A-C) Simplified schematic diagrams of looping events of heart, gut and stomach. (A) Heart looping. Two phases can be distinguished. First, the linear tube adopts S-shape. Outflow tract (yellow) and atrium (green) then move such that the outflow tract lies in front (ventral) and the atrium at the back (dorsal) of the ventricle, which is illustrated by arrows in the cross section taken at the level of the broken line. Inflow tract: orange; ventricle: blue. Left-sided expression of *Pitx2* in the linear heart is indicated in red. (B) Midgut. Looping occurs while it is located outside of the body in the so-called physiological umbilical hernia. The loop undergoes a 90° counterclockwise bend such that the two limbs lie next to each other. The future caecum becomes visible just distal to the apex of the midgut loop as a conical bud. When the physiological hernia retracts into the peritoneal cavity the caecum moves about 180° counterclockwise across the small intestine to complete the turning of the midgut. Small intestine: purple; large intestine: green. *Pitx2* expression is indicated in red. (C) Stomach. At E9.5 the spindle-shaped and still upright stomach rotates 90° counterclockwise such that the dorsal aspect (yellow) now points to the left. Between E10.5 and E12.5 the stomach rotates clockwise around a dorsoventral axis and comes to lie transversely across the abdominal cavity. Ventral aspect of stomach: green. (D-F) Left-sided *Pitx2* expression is maintained during heart looping. Heart of a E9.5 embryo in ventral (D), left (E) and right (F) view. at, atrium; ot, outflow tract; v, ventricle. (G-I) *Pitx2* expression at the sites of gut and stomach looping. (G) At E11.0 *Pitx2* mRNA is expressed at the apex of the turning midgut loop. Arrowheads mark position of physiological umbilical hernia. (H) Ventral view of the stomach of a E13.5 embryo. *Pitx2* mRNA expression is confined to the left side of the stomach (greater curvature). e, esophagus; li, large intestine; si, small intestine. (I) Stomach and intestines of a E16.5 embryo. The greater curvature of the stomach and the caecum stain positive for *Pitx2*. Note that the distal half of the caecum is positive whereas expression in the proximal part up to the junction with the small intestine (*) shows specific signals restricted to the outer curvature.

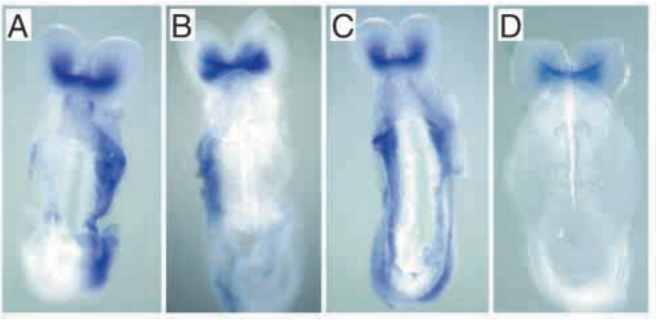


Fig. 5. Randomized LPM expression of *Pitx2* in *iv/iv* mutant mouse embryos. Whole-mount in situ hybridization of E8.5 *iv/iv* mutant embryos (8-12 somites) with a probe specific for mouse *Pitx2*. (A) Left-specific expression of *Pitx2*. (B) Right-specific expression of *Pitx2*. (C) Bilateral expression of *Pitx2*. (D) No expression of *Pitx2*.

was shown to randomize heart and gut situs upon RNA injection into right blastomeres of 16-cell *Xenopus* embryos (Hyatt and Yost, 1998). In order to test if such a treatment resulted in induction of *Pitx2* as well, we injected a CMV-*activin* DNA expression construct into right blastomeres at the 8-cell stage (Fig. 6C,C′). Dorsal-right injections resulted in bilateral *Pitx2* expression in 18% of embryos at stage 29 (*n*=22), while lateral-right injections about doubled this frequency (38%; *n*=16). These numbers correlate well with the heart and gut reversal rates reported by Hyatt and Yost (1998).

In order to analyze *Pitx2* induction in a quantitative way, we performed RT-PCR analysis of *Pitx2* mRNA in animal cap explants from untreated and growth factor injected embryos (Fig. 7). No induction was observed when the organizer-specific homeobox gene *goosecoid* (*gsc*) was injected. In contrast, both *Xnr1* and *activin* injection resulted in strong activation of *Pitx2* transcription in animal cap explants (Fig. 7), in agreement with the results obtained in injected whole embryos (Fig. 6B,C). Induction was markedly stronger with *Xnr1* as compared to *activin*. Activin treatment was efficient, as *Xnr1* was strongly induced (Fig. 7). The *Xenopus* homolog of another transcription factor with a localized expression pattern in the vertebrate heart, *XeHAND*, was recently shown to be induced in animal cap explants by the three TFGβ-like molecules BMP-2, BMP-4 and activin, although activin was only active at high doses (Sparrow et al., 1998). To test if TGFβ-like molecules in general promote *Pitx2* induction we injected BMP-4 and quantified *Pitx2* mRNA in animal cap explants by RT-PCR. BMP-4 was active in this assay, because the target gene *Xvent1* was induced, *Pitx2* RNA transcription, however, was not upregulated (Fig. 7). As *Xnr1* and *activin* both induce mesoderm in animal caps the specificity of *Pitx2* induction was further assessed by injecting FGF, which was a strong inducer of *XeHAND* as well (Sparrow et al., 1998). No *Pitx2* upregulation was observed, while the panmesodermal marker *Xbra* was strongly induced by FGF (Fig. 7). Our analysis shows that *Pitx2* mRNA was specifically induced by *Xnr1* and *activin* both in whole embryos and in animal cap explants, different from the induction of *Xenopus eHAND*. Together with our analysis of *Pitx2* and *nodal* expression in *iv/iv* mutant mouse embryos these data suggest that the TGFβ-like signaling molecule *nodal* could function as the endogenous inducer of *Pitx2* expression in the left LPM.

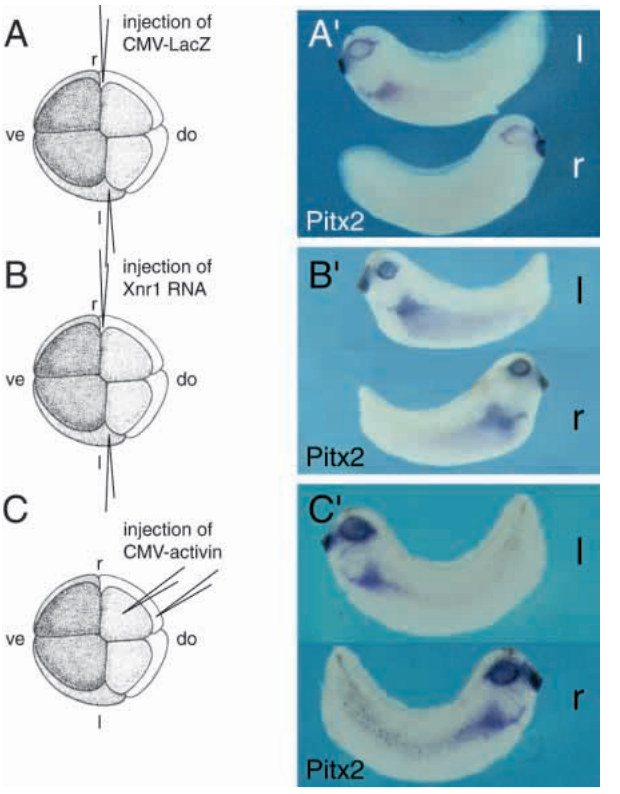


Fig. 6. Induction of *Pitx2* in the right LPM by *Xnr1* and *activin*. CMV-lacZ, CMV-*activin* and synthetic *Xnr1* mRNA were injected into defined blastomeres of *Xenopus* embryos at the 8-cell stage (A-C) and *Pitx2* transcripts were analyzed by whole mount in situ hybridization at stage 29 (A′-C′). (A,A′) Left-sided *Pitx2* expression in control injected embryos. (B,B′) *Pitx2* expression in the left and right LPM following bilateral *Xnr1* injection. (C,C′) Bilateral *Pitx2* expression following activin injection into dorsal-right blastomeres.

Misexpression of *Pitx2* causes laterality defects in *Xenopus* embryos

In order to assess a functional role for *Pitx2* in the process of generating laterality as well as in heart and gut morphology, we performed a series of misexpression experiments of *Pitx2* in *Xenopus*. Injections were performed into dorsal blastomeres because they are fated to become dorsolateral structures, including the prospective heart field, in later embryogenesis (Cleaver et al., 1996). Correct targeting was controlled by coinjection of a CMV-GFP construct (not shown).

Both laterality and morphology phenotypes were observed. Heart and gut defects arose together or separately. In the normal frog heart the ventricle is situated on the left side, with the outflow tract looping to the right side (Fig. 8B), and the gut coils counterclockwise (Fig 8D). Following injections into dorsal left blastomeres (*n*=11) most embryos developed normally (7/11; 64%), while a fraction displayed malformed hearts (2/11; 18%) or guts (2/11; 18%); inversion of situs was not observed. Upon injection into dorsal right blastomeres (*n*=22) two experimental embryos (9%) were normal with respect to heart and gut situs, while 32% showed situs

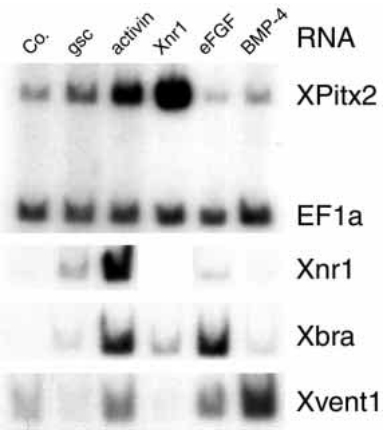


Fig. 7. Induction of *Pitx2* by *Xnr1* and *activin* in animal cap explants. Embryos were injected with *goosecoid*, *activin*, *Xnr1*, *eFGF* or *BMP-4* RNA into animal blastomeres at the 4-8 cell stage, animal caps were cut at stage 8, cultured until gastrula (stage 10.5-11), and assayed for the presence of *XPitx2*, *EF1-α*, *Xnr1*, *Xbra* and *Xvent1* RNA by RT-PCR. Note that *Pitx2* mRNA was specifically induced by *activin* and *Xnr1*. Co., control uninjected embryos.

inversion. Of these 18% displayed inversion of heart and gut, and 14% inversion of the heart alone. In the remaining embryos (59%) defects of heart (14%), gut (27%), or heart and gut (18%) morphologies were observed. Two examples for aberrant gut coiling are shown in Fig. 8F. The guts in these two embryos loop neither clockwise nor counterclockwise but stay more or less linear Fig. 8G shows one control (co) and three malformed hearts (e1-e3), which were characterized by a significant increase in size, particularly in the atrial region. In heart e2, for instance, the atrium was larger than the ventricle. In addition atrium, ventricle and outflow tract often were misaligned. The atrium of heart e1, for example, was located in a position slightly ventral to the ventricle, while in normal hearts the atrium lies dorsally to the ventricle.

Another series of injection experiments was performed using a mouse *Pitx2* clone. DNA injections resulted in a comparably low frequency of situs inversions and morphology phenotypes (<15%). When combinations of DNA and RNA were injected about half of the injected embryos showed situs inversion or aberrant heart and gut morphology. The very same types of malformed hearts and guts were observed as the ones reported here. However, in these experiments laterality defects were also observed upon injection into left blastomeres, most likely due to the use of RNA, as injections of high amounts of RNA resulted in strongly ventralized embryos (not shown).

DISCUSSION

Asymmetric expression of the homeobox gene *Pitx2* in the left LMP is a conserved feature between amphibian (*Xenopus*), fish (zebrafish) and mammalian (mouse) embryos, while the upstream signaling events may vary between species (King and Brown, 1997). Our functional analysis in *Xenopus* embryos strongly suggests that *Pitx2* functions as a mediator between signaling molecules which are only transiently active in the left LPM, such as *nodal* and *lefty-2*, and organs that assume

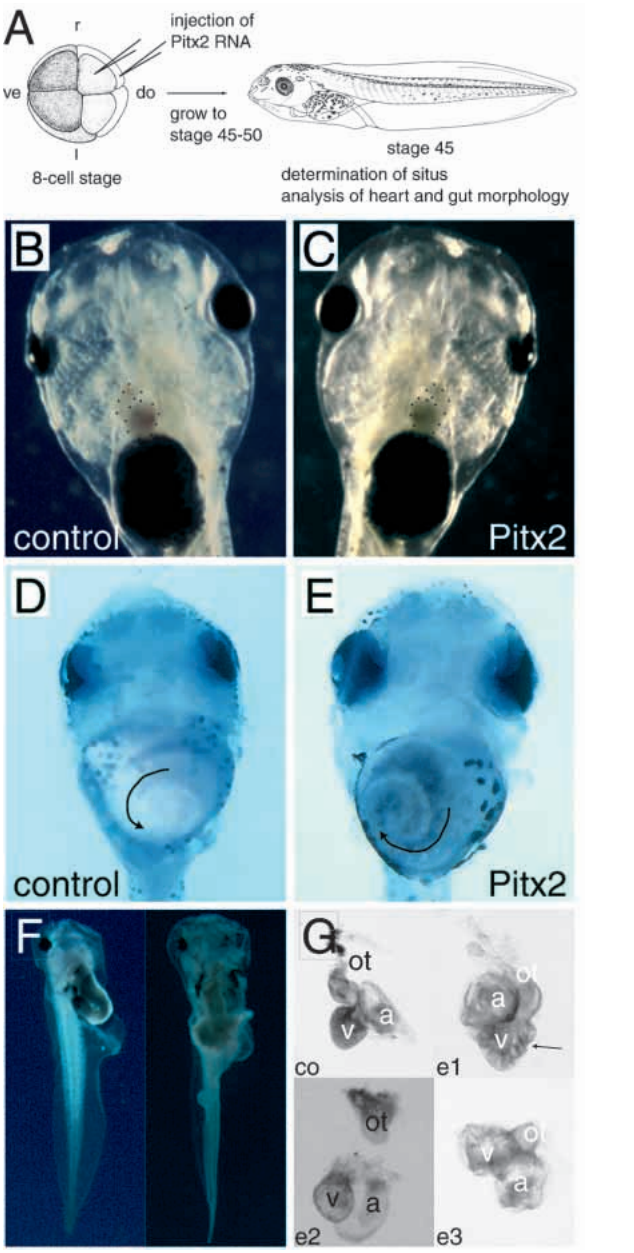


Fig. 8. Alterations of heart and gut laterality and morphology following misexpression of *Pitx2* in *Xenopus* embryos. (A) Experimental design. *Xenopus* embryos were injected at the 8-cell stage with a *Pitx2* DNA expression construct into both dorsal right blastomeres and cultured up to stage 45-50. Laterality of heart and gut was assessed in live embryos; heart morphology was analyzed following isolation of malformed hearts. (B-G) Ventral view of uninjected control embryos (B,D) with normal situs of the heart (B) and the gut (D) and *Pitx2*-injected specimen (C,E) displaying situs inversion of the heart (C) and the gut (E). Dots outline the shape of the heart in B and C; arrows indicate direction of gut looping in D and E. (F) Aberrant gut morphology of *Pitx2*-injected embryos. Note that the gut stayed almost linear in these two embryos. (G) Comparison of normal heart morphology (co) and three malformed hearts (e1-e3) resulting from *Pitx2* injections. Dorsal is to the right, and ventral to the left. The outflow tract in e2 was disconnected during preparation. Note that experimental hearts were hypertrophic and showed misalignment of atrium (a), ventricle (v) and outflow tract (ot).

asymmetric positions during embryogenesis, i.e. the heart and the gastrointestinal tract with its derivatives. While this manuscript was being prepared descriptive and functional studies of *Pitx2* in chick, mouse and *Xenopus* were published which support this conclusion (Logan et al., 1998; Piedra et al., 1998; Meno et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998).

Pitx2 and left-right signaling

Left asymmetric expression of *Pitx2* in mouse and frog embryos commences slightly later but in the same physical domain as that of the signaling molecule *nodal* (Fig. 2H). Misexpression of *Xnr1* in *Xenopus* in the right LPM induced *Pitx2* transcription in that region (Fig. 6B). In animal cap explants *Xnr1* functions as a strong inducer of *Pitx2* as well (Fig. 7). These data place *Pitx2* downstream of *nodal* in the left-right signaling cascade controling asymmetric morphogenesis. In addition, comparative analysis of expression patterns of *Pitx2* and *nodal* in *iv* mutant mice revealed the same four types of expression patterns for both genes, namely left, right, bilateral or absent, and almost identical frequencies of these patterns were observed (Fig. 3 and Table 1). The combined evidence of these experiments strongly suggests that *nodal* functions as the endogenous inducer of *Pitx2*.

Pitx2 and heart and gut looping

In contrast to the left-sided signaling molecules *nodal* and *lefty*, which are only transiently expressed in the left LPM, expression of *Pitx2* continues in the looping heart and gut (Figs 3, 4). Misexpression of *Pitx2* on the right side of the embryo resulted in inversion of heart and gut situs in about 30% of cases (Fig. 8), suggesting that *Pitx2* indeed plays a role in the process of organ looping. Situs inversion in *Xenopus* was also reported following misexpression of *Vg1*, *Xnr1* and *activin* on the right side of early embryos (Sampath et al., 1997; Hyatt and Yost, 1998). While *Vg1* presumably coordinates three-dimensional asymmetries through an interaction with the Spemann organizer (Hyatt and Yost, 1998), the effect of *activin*, like that of *Xnr1*, may well be mediated via *Pitx2*, as *activin* led to a strong induction of *Pitx2* mRNA transcription both in whole embryos (Fig. 6C) and in animal cap explants (Fig. 7).

We observed expression of *Pitx2* at a number of sites of differentiating smooth and skeletal muscle, such as the myocardium, muscular layer of the stomach, the myotome (not shown), eye and limb muscles (Fig. 6 and data not shown), and the body wall (Fig. 2C,D, and data not shown). It is therefore tempting to speculate that *Pitx2* might be involved in the transcriptional regulation of muscle-specific genes which in turn could be directly involved in asymmetric organ morphogenesis.

The phenotypes observed following *Pitx2* misexpression in the frog support that notion. In the majority of affected embryos heart and gut morphology showed aberrant features. In a number of cases the gut neither curled counterclockwise nor clockwise, but stayed more or less linear (Fig. 8F) or displayed aberrant looping (not shown). This phenotype may indicate a role of *Pitx2* in the biomechanics of gut looping. Malformed hearts were hypertrophic in most cases. Ventricles often appeared poorly trabeculated (Fig. 8G and data not shown), and atrium, ventricle and outflow tract displayed frequent misalignments (Fig. 8G, e1-e3). Looping of the heart has been attributed to differential proliferation (Stalsberg, 1969; Biben and Harvey, 1997). The aberrant growth seen in experimental hearts points to a possible role of *Pitx2* in the control of proliferation during heart morphogenesis. Misexpression of *Pitx2* in frog embryos thus resulted in situs inversion and/or aberrant organ morphology, in agreement with the expression of *Pitx2* both in the LPM and in the forming organs.

Two lines of evidence prove the specificity of the phenotypic alterations of laterality and organ morphology following misexpression of *Pitx2* in *Xenopus*. First, when *goosecoid* was misexpressed following the same protocol (injection of a DNA expression construct into dorsal right blastomeres at the 8-cell stage) we never observed any disturbances of laterality. The potential of *goosecoid* to affect dorsoanterior axis development upon misexpression on the ventral side has been well documented (De Robertis et al., 1992). Second, the heart and gut phenotypes, aberrant looping and hypertrophy, were never reported upon experimental alterations of laterality in *Xenopus*, such as interference with lateral signaling (Sampath et al., 1997; Hyatt and Yost, 1998) or with dorsoanterior or midline development (Danos and Yost, 1996; Nascone and Mercola, 1997; Sampath et al., 1997; Lohr et al., 1997).

Pitx2 and the human Rieger syndrome

Human *Pitx2* is mutated in Rieger syndrome, an autosomal dominant hereditary disease (Semina et al., 1996). This disorder is characterized by hypodontia, a protruding umbilical stump, and defects of the anterior eye chamber resulting in a high incidence of glaucoma (Jorgensen et al., 1978). The repositioning of the midgut loop into the abdominal cavity is brought about by an active shortening of the mesentery and contraction of the umbilical ring (Enblom, 1939), both of which express *Pitx2* (not shown). The association of Rieger syndrome with umbilical phenotypes and the more rare cardiac problems occasionally found in patients (Kulharya et al., 1995) provide evidence for a functional involvement of *Pitx2* in heart and gut development, despite the fact that no laterality defects were reported in Rieger patients. The mutations reported to date vary widely and include C-terminal truncations and point mutations in helices one, two or three of the homeo domain as well as splice mutations (Semina et al., 1996). They therefore most likely do not represent dominant gain-of-function mutations but rather argue that Rieger syndrome is caused by *Pitx2* haplo-insufficiency. Therefore, the right concentration of *Pitx2* protein seems to be crucial for correct physiological function of this transcription factor. Phenotypic effects on laterality and organ morphology may only be revealed upon complete loss of *Pitx2* function.

In conclusion, the asymmetric expression patterns of *Pitx2* in normal mouse, frog and zebrafish embryos, its randomized expression in the mouse laterality mutant *iv*, and the phenotypes obtained upon experimental manipulation of its expression domain in *Xenopus* embryos suggest that this gene mediates the transmission of a laterality signal from the left LPM to the primordia of the gastrointestinal tract and the heart.

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REFERENCES

Biben, C. and Harvey, R. P. (1997). Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene *eHAND* during murine heart development. *Genes Dev.* **11**, 1357-1369.

Blum, M., Gaunt, S. J., Cho, K. W. Y., Steinbeisser, H., Blumberg, B., Bittner, D. and De Robertis, E. M. (1992). Gastrulation in the mouse: the role of the homeobox gene goosecoid. *Cell* **69**, 1097-1106.

Chalmers, A. D. and Slack, J. M. W. (1998). Development of the gut in *Xenopus laevis*. *Dev. Dyn*. **212**, 509-521.

Cleaver, O. B., Patterson, K. D. and Krieg, P. A. (1996). Overexpression of the tinman-related genes Xnkx2. 5 and Xnkx-2. 3 in *Xenopus* embryos results in myocardial hyperplasia. *Development* **122**, 3549-3556.

Collignon, J., Varlet, I. and Robertson, E. J. (1996). Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature* **381**, 155-158.

Danos, M. C. and Yost, H. J. (1996). Role of notochord in specification of cardiac left-right orientation in zebrafish and Xenopus. *Dev. Biol.* **177**, 96-103.

Dareste, B. (1877). Recherches sur la production artificielle des monstruosités ou essais de tératogénie expérimentale. Reinwald, Paris.

De Robertis, E. M., Blum, M., Niehrs, C. and Steinbeisser, H. (1992). *goosecoid* and the Organizer. *Development* **Supplement**, 167-171.

Ding, X., Hausen, P. and Steinbeisser, H. (1998). Pre-MBT patterning of early gene regulation in *Xenopus*: the role of the cortical rotation and mesoderm induction. *Mech Dev.* **70**, 15-24.

Drysdale, T. A., Tonissen, K. F., Patterson K. D., Crawford M. J. and Krieg P. A. (1994). Cardiac troponin I is a heart-specific marker in the Xenopus embryo: expression during abnormal heart morphogenesis. *Dev. Biol.* **165**, 432-441.

Enbom, G. (1939). The early looping of the alimentary canal in the mammalian and human foetus and the mechanisms assumed to be active in this process. *Anat Rec* **75**, 409-414.

Gage, P. J. and Camper S. A. (1997). Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. *Hum. Mol. Gen.* **6**, 457-464.

Hanes, S. D. and Brent, R. (1989). DNA specificity of the bicoid activator protein is determined by homeodomain recognition helix residue 9. *Cell* **57**, 1275-1283.

Hummel, K. P. and Chapman, D. B. (1959). Visceral inversion and associated anomalies in the mouse. *J. Hered.* **50**, 9-13.

Hyatt, B. A. and Yost, H. J. (1998). The left-right coordinator: the role of Vg1 in organizing left-right axis formation. *Cell* **93**, 37-46.

Isaac, A., Sargent, M. G. and Cooke, J. (1997). Control of vertebrate left-right asymmetry by a snail-related zinc finger gene. *Science* **275**, 1301-1304.

Itasaki, N., Nakamura, H. and Yasuda, M. (1989). Changes in the arrangment of actin bundles during heart looping in the chick embryo. *Anat. Embryol.* **180**, 413-420.

Itasaki, N., Nakamura, H., Sumida, H. and Yasuda, M. (1991). Actin bundles on the right side in the caudal part of the heart tube play a role in dextro-looping in the embryonic chick heart. *Anat. Embryol*. **183**, 29-39.

Jones, C. M., Kuehn, M. R., Hogan, B. L. M., Smith, J. C. and Wright, C. V. E. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* **121**, 3651-3662.

Jorgenson, R. J., Levin, L. S., Cross, H. E., Yoder, F. and Kelly, T. E. (1978). The Rieger syndrome. *Am. J. Med. Genet.* **2**, 307-318.

King, T. and Brown, N. A. (1997). Embryonic asymmetry: left TGFB at the right time? *Curr. Biol.* **7**, 212-215.

Kitamura, K., Miura, H., Yanazawa, M., Miyashita, T. and Kato, K. (1997). Expression pattern of Brx1 (Rieg gene), Sonic hedgehog, Nk2. 2, Dlx1 and Arx during zona limithans intrathalamica and embryonic ventral lateral geniculate nuclear formation. *Mech. Dev.* **67**, 83-96.

Kulharya, A. S., Maberry, M., Kukolich, M. K., Day, D. W., Schneider, N. R., Wilson, G. N. and Tonk, V. (1995). Interstitial deletions 4q21. 1q25 and 4q25q27: phenotipic variability and relation to Rieger anomaly. *Am. J. Med. Gen.* **55**, 165-170.

Lamonerie, T., Tremblay, J. J., Lanctot, C., Therrien, M., Gauthire, Y. and Drouin, J. (1996). Ptx1, a bicoid-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene. *Genes Dev.* **10**, 1284-1295.

Lohr, J. L., Danos, M. C. and Yost, H. J. (1997). Left-right asymmetry of a nodal-related gene is regulated by dorsoanterior midline structures during *Xenopus* development. *Development* **124**, 1465-1472.

Logan, M., Pagan-Westphal, S. M., Smith, D. M., Paganessi, L. and Tabin, C. J. (1998). The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetricc signals. *Cell* **94**, 307-317.

Levin, M., Johnson, R., Stern, C., Kuehn, M. and Tabin, C. (1995). A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82**, 803-814.

Levin, M., Pagan, S., Roberts, D. J., Cooke, J., Kuehn, M. R. and Tabin, C. J. (1997). Left/right patterning signals and the independent regulation of different aspects of situs in the chick embryo. *Dev. Biol.* **189**, 57-67.

Lowe, L. A., Supp, D. M., Sampath K., Yokoyama, T., Wright, C. W., Potter, S. S., Overbeek, P. and Kuehn, M. R. (1996). Conserved left-right asymmetry of nodal expression and alterations in murine situs inversus. *Nature* **381**, 158-161.

Meno, C., Saijoh, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y, and Hamada, H. (1996). Left-right asymmetric expression of the TGFβ-family member *lefty* in mouse embryos. *Nature* **381**,151-155.

Meno, C., Shimono, A., Saijoh, Y., Yashiro, K., Mochida, K., Oishi, S., Noji, S., Kondoh, H. and Hamada, H. (1998). lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. *Cell* **94**, 287-297.

Moore, K. L. and Persaud, T. V. N. (1993). The developing human. Clinically oriented embryology. W. B. Saunderd editors, Philadelphia.

Mucchielli, M-l., Martinez, Salvador, Pattyn, A., Godiris, C. and Brunet, J-F. (1996). Otlx2, an otx-related homeobox gene expressed in the pituitary gland and in a restricted pattern in the forebrain. *Mol. Cell. Neurosc*. **8**, 252-271.

Nascone, N. and Mercola, M. (1997). Organizer induction determines left-right asymmetry in Xenopus. *Dev. Biol.* **189**, 68-78.

Nieuwkopp, P. D. and Faber, J. (1967). Normal table of *Xenopus laevis* (Daudin). Amsterdam: North-Holland publishing company.

Pagan-Westphal, S. M., and Tabin, C. J. (1998). The transfer of left-right positional information during chick embryogenesis. *Cell* **93**, 25-35.

Piedra, M. E., Icaardo, J. M., Albajar, M., Rodriguez-Rey, J. C. and Ros, M. A. (1998). Pitx2 participates in the late phase of the pathway controlling left-right asymmetry. *Cell* **94**, 319-324.

Price, R. L., Chintanowonges, C., Shiraishi, I., Borg, T. K. and Terracio, L. (1996). Local and regional variations in myofibrillar patterns in looping rat hearts. *The Anat. Rec.* **245**, 83-93.

Rupp, R A. W., Snider, L. and Weintraub, H. (1994). Xenopus embryos regulate the nuclear organization of XMyoD. *Genes Dev.* **8**, 1311-1323.

Ryan, A. K., Blumberg, B., Rodriguez-Esteban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., de la Pena, J., Sabbagh, W., Greenwald, J., Choe, S., Norris, D. P., Robertson, E. J., Evans, R. M., Rosenfeld, M. R. and Izpisua-Belmonte, J. C. (1998). Pitx2 determines left-right asymmetry of internal organs in vertebrates. *Nature* **394**, 545-551.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbour Laboratory Press, New York, Cold Spring Harbor Laboratory.

Sampath, K., Cheng, A. M. S., Frisch, A. and Wright, C. V. E. (1997). Functional differences among *Xenopus* nodal-related genes in left-right axis determination. *Development* **124**, 3293-3302.

Schaart, G., Viebahn, C., Langmann, W. and Ramaekers, F. C. S. (1989). Desmin and titin expression in early postimplantation mouse embryos. *Development* **107**, 585-596.

Semina, E. V, Reiter, R., Leysens, N. J., Alward, W. L. M., Small, K. W., Datson, N. A, Siegel-Bartelt, J., Bierke-Nelson, D., Bitoun, P., Zabel, B. U., Carey, J. C. and Murray, J. C. (1996). Cloning and characterization of a novel bicoid- related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nat. Genet.* **14**, 392-399.

Sparrow, D. B., Kotecha, S., Towers, N. and Mohun, T. J. (1998). *Xenopus eHAND*: a marker for the developing cardiovascular system of the embryo is regulated by bone morphogenetic proteins. *Mech Dev*. **71**, 151-163.

Srivastava, D., Cserjesi, P. and Olson, E. N. (1995). A subclass of bHLH proteins required for cardiac morphogenesis. *Science* **270**, 1995-1999.

Stalsberg, H. (1969). The origin of heart asymmetry: Right and left contributions to the early chick embryo heart. *Dev. Biol.* **19**, 109-129.

Tsuda,T., Philp, N., Zile, J. K. and Linask, K. K. (1996). Left-right asymmetric location of flectin in the extracellular matrix during heart looping. *Dev. Biol*. **173**, 39-50.

Vorbrüggen, G., Constien, R., Zilian, O., Wimmer, E. A., Dowe, G., Taubert, H., Noll, M. and Jäckle, H. (1997). Embryonic expression and characterization of a Ptx1 homolog in Drosophila. *Mech. Dev.* **68**, 139-147

Wilhelmi, H. (1921). Experimentelle Untersuchungen über Situs inversus viscerum. *Arch. EntwMech. Org.* **48**, 517-532.

Yoshioka, H., Meno, C., Koshiba, K., Sugihara, M., Itoh, H., Ishimura, Y., Inoue, T., Ohuchi, H., Semina, E., Murray, J. C., Hamada, H. and Noji, S. (1998). Pitx2, a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. *Cell* **94**, 299-305.