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 (Darest, 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; Schaert 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
Ptis2-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)GIC(G/T)(A/G)TT(C/T)TT(A/G)AACCAIA(A/G)(A/C)GIACIA T(A/C/T)TT(C/T)AC and 5′G6CCG(C/T)(C/G)T(G/T)(A/G)TT(C/T)TT(Xenopus)(A/G)AACCAIA(A/G)(A/C)GIC). 50 ng cDNA specific for the -specific sequences were cloned following RT-PCR from P19 clones (XPitx2). Full length cDNA clones were obtained by screening a neurula cDNA library (stage 18, Stratagene) with the entire coding region of mouse Pitx2. Full length cDNA clones were isolated from mouse, Xenopus and zebrafish Pitx2 cDNA clones using degenerate primers following standard procedures (Sambrook et al., 1989). A 641 bp fragment of zebrafish Pitx2 cDNA was cloned by RT-PCR from polyadenylated RNA isolated from 15- to 20-somite embryos using 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 Xhol-EcoRI fragment corresponding to entire coding sequence plus 5′ and 3′ UTR), Xenopus Pitx2 (456 bp PstI fragment, corresponding to nucleotides 316-796 of the sequence submitted to the database; accession number AJ005787), Xenopus cardiac troponin I (927 bp NotI-EcoRI fragment; Drysdale et al., 1994), Xnr1 (1515 bp EcoRI-Xhol 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 0.5x MBSS until control embryos reached stage 10.5-11 (gastrula). Total RNA was extracted using a commercial kit (Tristar, AFS) 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 GTCTGGGGAGTGTAAGCTAAG, reverse TGGTGAGCGAG-TAAGCTGGGTTAC, 29 cycles at 30 seconds/95°C, 30 seconds/57°C, 30 seconds/72°C; EF1α forward CATAGTGTGCTGGATAGC, reverse ACTGCGTGGACTCTCTAG, 24 cycles at 30 seconds/95°C, 30 seconds/57°C, 30 seconds/72°C; Xnr1 forward AGTCAGCTTCTGCAAGCC, reverse TCAAAAACACCTCATA-CTCCC, 26 cycles at 30 seconds/95°C, 30 seconds/53°C, 30 seconds/72°C; Xbra forward CACAGTCTATCGACTGACCC, reverse TTCTGTGAGTGACGAGTTC, 26 cycles at 30 seconds/95°C, 30 seconds/57°C, 30 seconds/72°C; Xvent1 forward ATCGACCTTCTGATCTCCTCGTC, reverse CACCAGCCGG-CTGAAACCGCATTC, 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 immunochemistry 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), Otx2 (Mucchielli et al., 1996), Brx1a (Kitamura et al., 1997), and Pitx2 (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, Vorbüringen 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...
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 homzygous iv animals display situs solitus, whereas the other half show situs inversus.

Homzygous 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.

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,

Table 1. Pitx2 and nodal expression versus sidedness

<table>
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<th>Gene</th>
<th>Left</th>
<th>Right</th>
<th>Both</th>
<th>None</th>
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<td>nodal (29)</td>
<td>7 (24%)</td>
<td>6 (21%)</td>
<td>10 (34%)</td>
<td>6 (21%)</td>
</tr>
<tr>
<td>Pitx2 (32)</td>
<td>9 (28%)</td>
<td>8 (25%)</td>
<td>10 (31%)</td>
<td>5 (16%)</td>
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Compilation of expression patterns of Pitx2 and nodal in iv/iv mutant embryos at 5-9 somites analyzed by whole-mount in situ hybridization.
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 (Nieuwkoop 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.
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 TFGβ-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 pannesodermal 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.

**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
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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 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.

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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).

**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.

**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 un.injected 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 controlling 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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