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# Mesendoderm and left-right brain, heart and gut development are differentially regulated by *pitx2* isoforms

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## SUMMARY

**The *pitx2* gene is a member of the *bicoid*-homeodomain class of transcription factors that has been implicated in the control of left-right asymmetry during organogenesis. Here we demonstrate that in zebrafish there are two *pitx2* isoforms, *pitx2a* and *pitx2c*, which show distinct expression patterns and have non-overlapping functions during mesendoderm and asymmetric organ development. *pitx2c* is expressed symmetrically in presumptive mesendoderm during late blastula stages and in the prechordal plate during late gastrulation. *pitx2a* expression is first detected at bud stage in the anterior prechordal plate. The regulation of early mesendoderm *pitx2c* expression is dependent on *one-eyed pinhead* (EGF-CFC-related gene) and *spadetail* (*tbx*-transcription factor) and can be induced by ectopic *goosecoid* expression. Maintenance of *pitx2c* midline expression is dependent on *cyclops* (*nodal*) and *schmalspur*, but not *no tail* (*brachyury*). Ectopic expression of *pitx2* isoforms results in distinct morphological and molecular phenotypes, indicating that *pitx2a* and *pitx2c* have divergent regulatory functions. Both isoforms downregulate *goosecoid* on the dorsal side, but in contrast to earlier reports that *nodal* and *lefty* are upstream of *pitx2*, ectopic *pitx2c* in other regions induces *cyclops*, *lefty2* and *goosecoid* expression.**

**Asymmetric isoform expression occurs in non-overlapping domains, with *pitx2c* in left dorsal diencephalon and developing gut and *pitx2a* in left heart primordium. Targeted asymmetric expression in *Xenopus* shows that both isoforms can alter left-right development, but *pitx2a* has a slightly stronger effect on heart laterality. Our results indicate that distinct genetic pathways regulate *pitx2a* and *pitx2c* isoform expression, and each isoform regulates different downstream pathways during mesendoderm and asymmetric organ development.**

Key words: *pitx2*, *lefty*, *nodal*, *cyclops*, *one-eyed pinhead*, Mesoderm, Endoderm, Left-right development

## INTRODUCTION

The three primary tissue layers, endoderm, mesoderm and ectoderm, are defined during vertebrate gastrulation. Of the three, the origin and patterning of the most internal layer, the endoderm, is least understood. At late blastula stages during zebrafish embryogenesis, the blastoderm begins to thin and spread over the large yolk cell in a process termed epiboly. Marginal cells located around the entire circumference of the blastoderm are fated to become both mesoderm and endoderm during gastrulation (Kimmel et al., 1990). As determined by fate mapping, the mesoderm domain extends more cell-diameter lengths from the blastoderm margin than the endoderm; the cells closest to the yolk correspond to both presumptive mesoderm and endoderm (Warga and Nusslein-Volhard, 1999). During gastrulation, dorsal cells in the embryonic shield involute to form the notochord and the mesendoderm of the prechordal plate (Shih and Fraser, 1995). The endoderm component of the prechordal plate later contributes to the internal organs, and the mesoderm forms the hatching gland and head mesoderm. Signals from the shield and subsequent midline, including the prechordal plate, are responsible for patterning the embryo along the anterior-posterior, dorsal-ventral and left-right axes (Chen et al., 1997; Schier and Talbot, 1998).

*Pitx2* is a member of the *bicoid*-class of *paired* homeodomain transcription factors that is mutated in patients with Reiger syndrome (Semina et al., 1996). *Pitx2* has also been implicated in late stages of left-right development and is expressed bilaterally in anterior mesendoderm and on the left side in the lateral plate mesoderm, which forms the cardiac and visceral primordia in mouse (Meno et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998), chick (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; St Amand et al., 1998; Yoshioka et al., 1998), frog (Campione et al., 1999; Ryan et al., 1998) and zebrafish (Campione et al., 1999). Isoforms of human *pitx2* are expressed by alternative splicing and the use of two promoters (Arakawa et al., 1998; Semina et al., 1996), generating proteins identical throughout their homeodomains and carboxy termini but different in their amino termini. Previous developmental studies have not made distinctions among *pitx2* isoforms, and neither the developmental upstream regulatory pathways nor downstream functions of *pitx2* isoforms are known. Here we identify *pitx2*

isoforms in zebrafish and examine their differential expression and functions during development of the mesendoderm, prechordal plate and left-right asymmetry.

Mesendoderm patterning appears to involve complex inductive and antagonistic interactions. In zebrafish, mutations in *cyclops* (*cyc*) (Hatta et al., 1991), *squint* (*sqt*) (Heisenberg and Nusslein-Volhard, 1997) and *one-eyed pinhead* (*oep*) (Hammerschmidt et al., 1996; Schier et al., 1996) lead to cyclopia and demonstrate a requirement for these genes in formation of the prechordal plate. Cyc and Sqt are members of the *nodal*-related *transforming growth factor β* (TGFβ) cell-cell signaling molecule family (Feldman et al., 1998; Rebagliati et al., 1998b; Sampath et al., 1998), while *oep* encodes a novel EGF-CFC transmembrane protein that appears to mediate both Cyc and Sqt signaling (Gritsman et al., 1999; Zhang et al., 1998). Conversely, other members of the TGFβ family, the zebrafish *lefty*-related genes (*lft1* and *lft2*), are expressed in the same mesendoderm precursors in zebrafish but are antagonistic to *nodal*-related signaling (Bisgrove et al., 1999; Meno et al., 1999; Thisse and Thisse, 1999). Ectopic expression of either *lft1* or *lft2* in zebrafish also produces cyclopia, suggesting that a balance between Lft and Cyc/Sqt/Oep signaling is required for proper prechordal plate development and ventral patterning of the central nervous system (CNS).

The interactions of *pitx2* isoforms with genes implicated in mesendoderm development are examined here. The isoform expressed in early mesendoderm, *pitx2c*, is regulated by TGFβ signaling that controls prechordal plate development, including *cyc* and *oep*. In addition, increased expression of *pitx2c* induces *cyc* expression, suggesting a novel feedback mechanism on *cyc* by Pitx2c. Early ectopic expression of *pitx2a* or *pitx2c* disrupts gastrulation with the isoforms regulating their own and cross-regulating each other's expression.

In addition to early roles in mesendoderm patterning, *cyc*, *lft1* and *lft2* are expressed asymmetrically in zebrafish during late somite stages in the left dorsal diencephalon and left lateral plate mesoderm (Bisgrove et al., 1999; Rebagliati et al., 1998a; Sampath et al., 1998; Thisse and Thisse, 1999). Surprisingly, *pitx2* isoforms are differentially expressed in these left primordia. Targeted ectopic expression assays in *Xenopus* embryos show that both Pitx2a and Pitx2c are competent to randomize left-right development; however, Pitx2a has a greater impact on cardiac asymmetry than Pitx2c. Taken together, these results show differential regulation and distinct functions of *pitx2* isoforms during both early mesendoderm development and later left-right development.

## MATERIALS AND METHODS

### Embryo culture and zebrafish strains

Zebrafish embryos were obtained by natural spawning and maintained at 28.5°C in system water (Westerfield, 1995). Fish heterozygous for *cyclops* (*cyc$^{b229}$* and *cyc$^{tf219}$*) (Brand et al., 1996; Hatta et al., 1991), *iguana* (*igu$^{ts294e}$*) (Brand et al., 1996), *no tail* (*ntl$^{b195}$*) (Halpern et al., 1993), *one-eyed pinhead* (*oep$^{m134}$*) (Schier et al., 1996), *schmalspur* (*sur$^{ty68b}$*) (Brand et al., 1996) and *spadetail* (*spt$^{b104}$*) (Kimmel et al., 1989) were bred to obtain homozygous mutant embryos.

### Isolation of *pitx2* isoforms in zebrafish

A 500 bp fragment with identity to *pitx2* from other species was amplified by degenerate primer-based PCR using 24 hours post-fertilization (hpf) embryo cDNA as a template. Degenerate primers were designed using the CODEHOP program (Rose et al., 1998): 5′-CCC GGG AGG AGA TCG CNG TNT GGA C-3′ and 5′-TGC AGG TGT CCC GGT ACA CRT ANG GNG G-3′. Total RNA was isolated from zebrafish embryos using Trizol (BRL) and further purified using Qiagen RNeasy minicolumns. cDNA templates for PCR were reverse-transcribed from RNA using oligo d(T) and Superscript II reverse transcriptase (BRL). The PCR product was amplified with Platinum *Taq* (BRL) and cloned into the vector pCRII using the TOPO TA cloning kit (Invitrogen). This fragment was used to screen a late somite cDNA library (a generous gift from David Grunwald, University of Utah) at reduced stringency: 30% formamide, 5× SSPE (Sigma), 5× Denhardt's solution, 0.5% SDS, 100 μg/ml yeast RNA (Sigma) at 42°C. Membranes were washed at a final stringency of 2× SSPE/0.1% SDS at 50°C. Fifteen positive clones were subjected to restriction enzyme analysis and sequencing and placed into two groups corresponding to *pitx2a* and *pitx2c*.

### Northern blots

Total RNA from staged embryos was isolated as above, and 5 μg from each stage were treated with glyoxal (Ausubel et al., 1987) for 1 hour at 50°C. The RNA was fractionated in a 1% agarose gel in 1× TAE and transferred with 20× SSC. Isoform specific probes for *pitx2a* (5′-*pitx2a*) and *pitx2c* (5′-*pitx2c*) were generated from the 5′ untranslated regions (UTRs). The 400 bp amplification fragment corresponding to the 5′ UTR from *pitx2a* was cloned using 5′ RACE-PCR (BRL) with the following oligonucleotides for reverse transcription (RT) and PCR: RT, 5′-TTC TCT AAT TGA GCA CAC GTT GAT GC-3′; PCR, 5′-CAC GTT GAT GCA AGT TTG CGG-3′. A 5′-UTR probe to *pitx2c* was generated by subcloning a 400 bp *Bam*HI restriction fragment from the full-length *pitx2c* cDNA into pBSIIKS– (Stratagene). DNA probes were labeled by random priming in the presence of [α-$^{32}$P]dCTP using the Decaprime kit (Ambion). Hybridizations were carried out using Quik Hyb (Stratagene) following the manufacturer's instructions.

### In situ hybridization

A full-length *pitx2a* probe was used to detect all mRNAs expressed from the *pitx2* gene by in situ hybridization. Isoform-specific probes were the same as used above for northern hybridizations. Probes generated from cDNAs corresponding to *gsc* (Stachel et al., 1993), *no tail* (Schulte-Merker et al., 1992), *cyc* (Rebagliati et al., 1998a) and *lft2* (Bisgrove et al., 1999) were used as molecular markers. Probes were produced by transcription from linear templates using the Maxiscript kit (Ambion) in the presence of either digoxigenin-11-UTP or fluorescein-12-UTP (BMB). Free nucleotides were removed using Bio-Gel P-6 Micro Bio Spin columns (BioRad).

Embryos were fixed in 4% paraformaldehyde in sucrose buffer (Westerfield, 1995) overnight at 4°C. In situ hybridizations were carried out at 70°C (Essner et al., 1996). Anti-digoxigenin- or anti-fluorescein-alkaline phosphatase (AP)-conjugated antibodies (BMB) were used at a 1:2000 dilution for detection of digoxigenin- and fluorescein-labeled probes, respectively. For double in situ hybridizations, probes were hybridized simultaneously and detected using sequential alkaline phosphatase reactions. After development of the first probe with NBT and BCIP (Sigma), the anti-digoxigenin antibody was removed by incubating the embryos in 100 mM glycine, pH 2.2 for 20 minutes followed by three washes in PBS with 0.1% Tween 20 (PBST). Embryos were refixed overnight in 4% paraformaldehyde in PBS. An anti-fluorescein-AP antibody against the second probe was absorbed to the embryos and detected with Fast Red (Sigma). Embryos were refixed, cleared in either 70% glycerol in PBS or 2:1 benzylbenzoate:benzyl alcohol and photographed with a Leica MZ12 stereoscope.

### *pitx2* and *gsc* constructs and ectopic expression

For construction of expression vectors, the coding sequences of

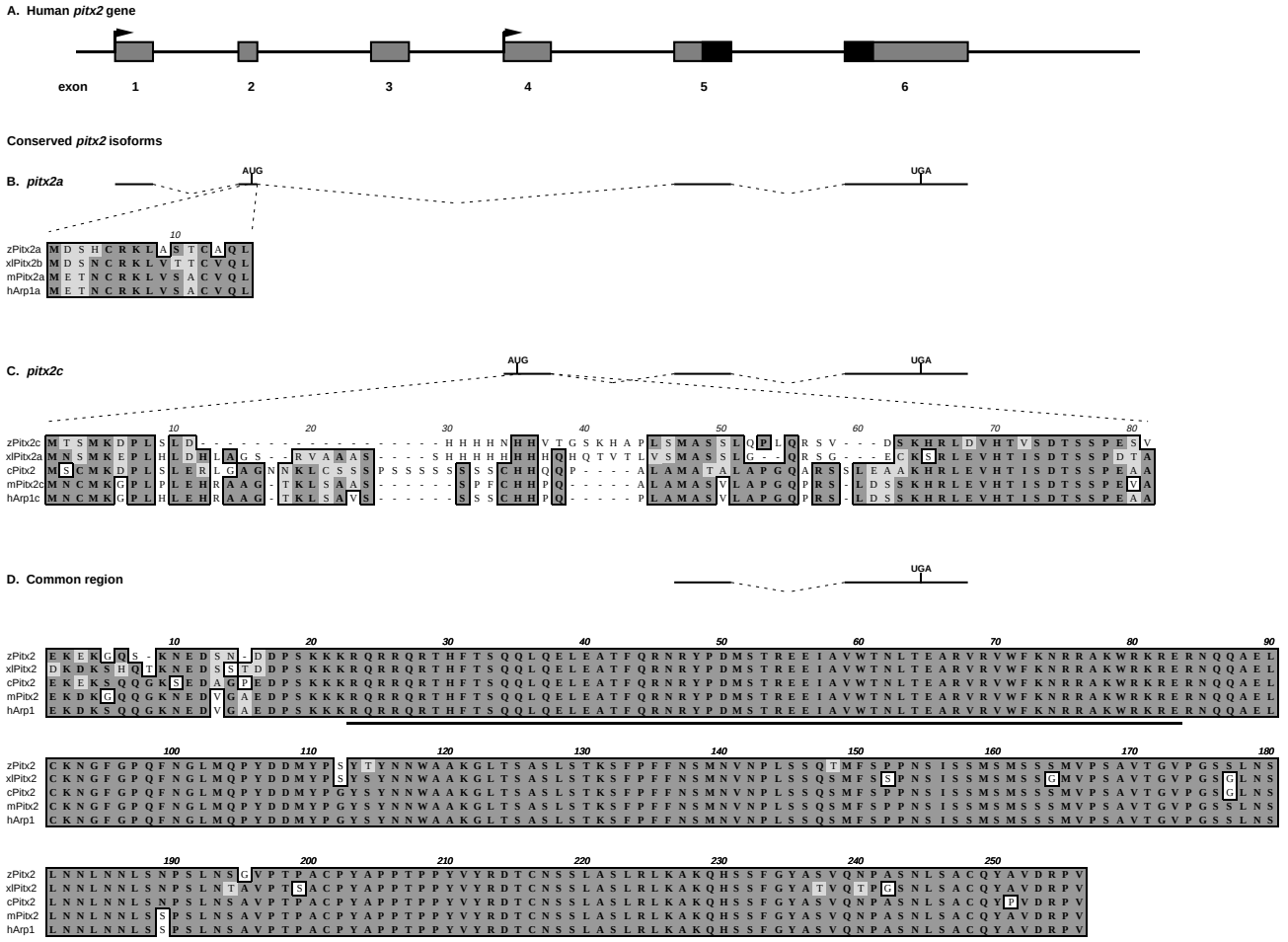


**Fig. 1.** *pitx2* isoforms are conserved in vertebrate evolution. (A) The human *pitx2* gene uses two promoters (arrows) to generate *pitx2a* and *pitx2c* isoforms that differ in their coding potential in 5′ regions (Arakawa et al., 1998). The zebrafish genomic structure is unknown. Here, the Pitx2 proteins predicted from cDNA sequences are compared across vertebrate species; conservation of the amino acid sequence for both zebrafish isoforms suggests a genomic structure similar to that in humans. Black boxes represent regions encoding homeodomain. (B) The zebrafish (z) Pitx2a unique region compared to Pitx2a from mouse (m), the human (h) Pitx2a homolog, Arp1a, and *Xenopus* (xl) Pitx2b. The nomenclature of the *Xenopus* isoforms does not conform with the nomenclature of other species. (C) The zebrafish Pitx2c unique region compared to a similar region in other species including chicken (c). (D) The *bicoid*-like homeodomain and C-terminal region of zebrafish Pitx2 compared to Pitx2 from other species. The homeodomain region is underlined. Dark gray boxes show regions of identity, while light gray show conservative amino acid substitutions.

*pitx2a*, *pitx2c* and *gsc* were PCR amplified with *Pfu* polymerase (Stratagene). The PCR products were restriction-digested and directionally ligated into pCS2+, a vector that allows both the production of in vitro synthesized transcripts and in vivo DNA expression in embryos from a CMV promoter and enhancer (Rupp et al., 1994; Turner and Weintraub, 1994). The cloned cDNA fragments were sequenced to confirm identity and integrity.

For injection of synthetic mRNAs into early zebrafish embryos, linear templates from pCS2+/*pitx2a* and *pitx2c* were produced for transcription. The templates were transcribed in vitro with SP6 RNA polymerase using the Message Machine transcription kit (Ambion). The concentrations of the RNAs were adjusted to inject approximately 5 pg of RNA for *pitx2a* and *pitx2c* and 1 pg for *gsc* per zebrafish embryo. Embryos were injected at the 1- to 2-cell stage.

For targeted left-right asymmetric expression of zebrafish *pitx2a* and *pitx2c* in *Xenopus* embryos, either pCS2+/*pitx2a* or *pitx2c* DNA was injected at the future margin of either the ventral left or ventral right blastomere of 4-cell embryos (stage 3) (Nieuwkoop and Faber, 1967). Approximately 15-25 pg of either *pitx2* DNA was coinjected with 15-25 pg of pEGFP-N1 DNA (Clonetech), and the correct right or left localization of GFP expression was confirmed using epifluorescence illumination of living embryos between stages 43-46. Correctly targeted embryos that did not display dorsoventral or anteroposterior defects were scored for heart and gut morphologies at stage 46.

### Immunocytochemistry with *Xenopus* embryos

For antibody staining, *Xenopus* embryos were fixed at stage 46 in 4% paraformaldehyde/PBS overnight at 4°C and subsequently rinsed and stored in PBS. Embryos were dehydrated with a series of methanol washes and placed at –20°C in 100% methanol overnight. After rehydration, embryos were washed with PBST and blocked in 5% sheep serum and 2% bovine serum albumin (BSA) in PBST at room temperature. Monoclonal antibodies against bovine cardiac troponin T (DSHB University of Iowa) were diluted 1:1 with blocking solution and absorbed to embryos overnight at 4°C. Embryos were washed with 2% BSA in PBST at room temperature. Fluorescein-conjugated, goat anti-mouse IgG2a (Southern Biotechnology Associates, Inc.) secondary antibodies were diluted 1:100 in blocking solution and incubated with embryos overnight at 4°C. Embryos were washed with

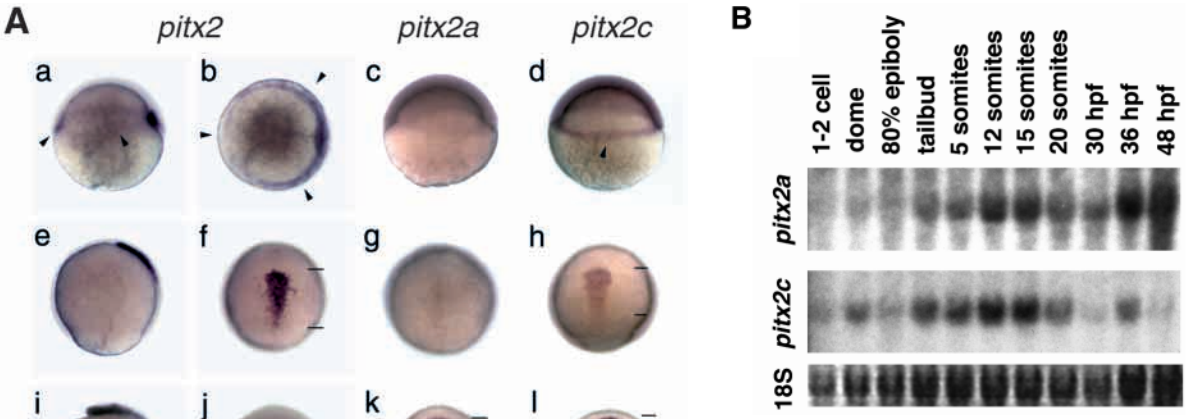


**Fig. 2.** (A) *pitx2c* is expressed in the early mesendoderm and prechordal plate, and *pitx2a* is expressed late in the anterior prechordal plate (polster). Expression was detected by in situ hybridization. (a,b,e,f,i,j,m,n) A full-length *pitx2* probe was used to detect all *pitx2* transcripts. (c,g,k,o) An isoform-specific 5′-UTR probe from *pitx2a*. (d,h,l,p) An isoform-specific 5′-UTR probe from *pitx2c*. (a-d) 40-50% epiboly: (a,b) *pitx2* expression was observed in marginal blastomeres (arrowheads) with increased levels on the dorsal side. (c) No expression of *pitx2a* was detected. (d) Expression of *pitx2c* in marginal blastomeres (arrowhead). (e-h) 90% epiboly: (e,f) *pitx2* expression in the prechordal plate. (g) No expression of *pitx2a* was detected. (h) *pitx2c* expression in the prechordal plate. (i-l) bud stage: (i,j) *pitx2* expression in the polster and posterior prechordal plate. (k) *pitx2a* expression in the polster. (l) *pitx2c* expression in the polster and posterior prechordal plate. (m-p) 4-6 somites: (m,n) *pitx2* expression was observed in both the polster and posterior prechordal plate. (o) *pitx2a* expression in the polster. (p) *pitx2c* expression in the polster and the posterior prechordal plate. In a,c,d,e,i,m, embryo views are lateral with dorsal to the right. In b, animal pole view is shown with dorsal to the right. In f-h, j-l and n-p, dorsoanterior views are shown with anterior at the top. Bars indicate the extent of expression in the prechordal plate along the anteroposterior axis. (B) Northern blot hybridizations using isoform-specific 5′-UTR probes on RNA isolated from staged embryos. The same blot was used in all panels. Ethidium bromide staining of 18S ribosomal RNA is shown at the bottom as a loading control.

2% BSA in PBST and cleared in 70% glycerol containing Slow Fade (Molecular Probes). Embryos were visualized at 5× magnification using epifluorescence on a Leica DMR microscope and photographed using a DAGE color video camera.

## RESULTS

### Characterization and expression of *pitx2* isoforms in zebrafish

To understand the conservation of *pitx2* during vertebrate evolution and its involvement in head and asymmetric organ development, we used degenerate RT-PCR to clone *pitx2* from late somite stage zebrafish embryos. A 500 bp fragment with identity to *pitx2* from other vertebrate species was cloned and used to screen a 20-28 hpf stage library. Two classes of clones corresponding to two different isoforms of *pitx2* from humans and mice were recovered and designated zebrafish *pitx2a* and *pitx2c* (GenBank accession numbers: AF132447 and AF132446). These isoforms are identical in their 3′-UTRs and most of their coding sequences; however, they differ in their 5′-UTRs and N-terminal coding sequences (Fig. 1). In cultured

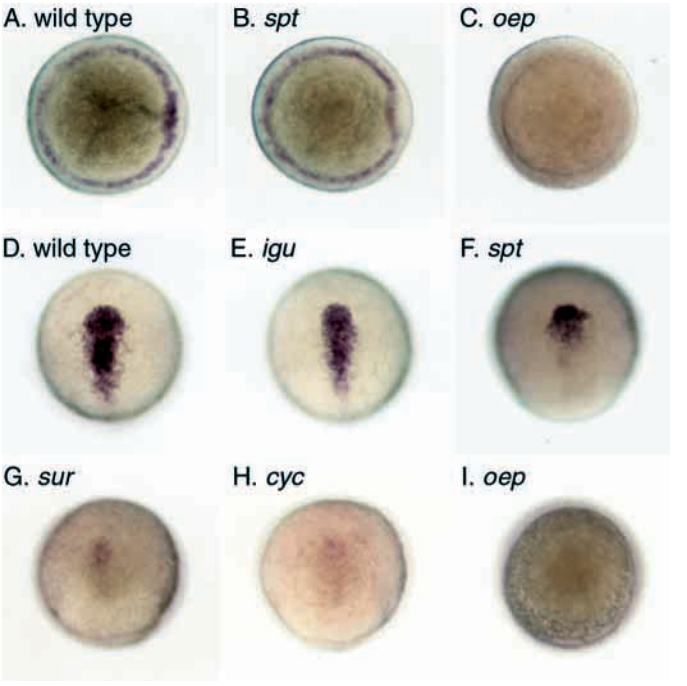


**Fig. 3.** Mesendoderm expression of *pitx2c* is dependent on *spt*, *oep*, *sur* and *cyc*. In situ hybridizations using a full-length *pitx2* probe are shown. (A-C) Mesendoderm expression of *pitx2* at 50% epiboly. (A) Wild-type embryo. (B) *spt* and (C) *oep* mutant embryos. Animal pole views are shown with dorsal to the right. (D-I) Dorsoanterior views with the anterior at the top showing the prechordal plate expression of *pitx2* at 80-90% epiboly. (D) Wild-type embryo. (E) *igu*, (F) *spt*, (G) *sur*, (H) $cyc^{b229}$ and (I) *oep* mutant embryos.

human cell lines, different *pitx2* isoforms arise from utilization of both different promoters and alternative splicing (Arakawa et al., 1998; Semina et al., 1996). The human *pitx2a* and *pitx2b* isoforms represent transcription from the same promoter and differ by alternative splicing of exon 2, while the *pitx2c* isoform is generated from a separate promoter. While the genomic structure of the zebrafish *pitx2* gene is not known, the encoded zebrafish Pitx2a protein displays 60-73% identity to Pitx2a from *Xenopus* (Campione et al., 1999), mice (Gage and Camper, 1997) and humans (Arakawa et al., 1998) over 15 N-terminal amino acids unique to Pitx2a (Fig. 1B). The zebrafish Pitx2c protein shows a lower amino acid identity (38-50%) to that of *Xenopus* (Campione et al., 1999), chicken (St Amand et al., 1998), mice (Arakawa et al., 1998) and humans (Arakawa et al., 1998) over a 61-amino-acid region unique to Pitx2c (Fig. 1C). The remainder of the zebrafish Pitx2 protein, common to all isoforms and containing the *bicoid*-like homeodomain, displays approximately 95% identity to Pitx2 from other species (Fig. 1D). The high degree of structural conservation among *pitx2* isoforms suggests conserved functional roles during development.

To define the potential roles of the zebrafish *pitx2* isoforms during early development, *pitx2* expression patterns were determined by in situ hybridization. Hybridizations were carried out with both a full-length *pitx2* probe in order to detect all *pitx2* transcripts and unique 5′-UTR probes to characterize isoform-specific expression (Fig. 2A). Expression of *pitx2* was observed during dome stage to 60% epiboly in a ring of marginal cells around the entire circumference of the embryo (Fig. 2Aa-d). A similar hybridization pattern was observed with both the *pitx2c*-specific probe and the full-length *pitx2* probe. Interestingly, the marginal cells expressing *pitx2c* were found only 4 cell-diameter lengths from the margin during dome to 40% epiboly (data not shown). These cells give rise to both endoderm and mesoderm while cells further from the margin give rise to mesoderm only (Warga and Nusslein-Volhard, 1999). By 60% epiboly *pitx2c*-expressing cells had a deep location in the blastoderm next to the yolk cell, suggesting an endodermal phenotype (data not shown). At 40% epiboly an increased number of cells on the dorsal side accumulated *pitx2c* transcripts. During gastrulation, dorsal marginal cells involute and form the prechordal plate and notochord (Kimmel et al., 1995). *pitx2c* was detected in both the anterior (polster) and posterior prechordal plate mesendoderm at 90% epiboly (Fig. 2Ae-h). During these early phases of *pitx2* expression, *pitx2a* was not detected (Fig. 2Ac,g). However, at bud stage following gastrulation, *pitx2a* was observed in the polster and *pitx2c* in both the polster and posterior prechordal plate mesendoderm (Fig. 2Ai-l). At 4- to 6-somite stages, *pitx2a* and *pitx2c* expression was detected in the polster (Fig. 2Am-p). Additionally, weak *pitx2c* expression remained in the posterior prechordal plate (Fig. 2Ap). As detected with the full-length *pitx2* probe, *pitx2a* transcripts were also observed in lateral head mesendodermal cells extending from the polster (Fig. 2An,o).

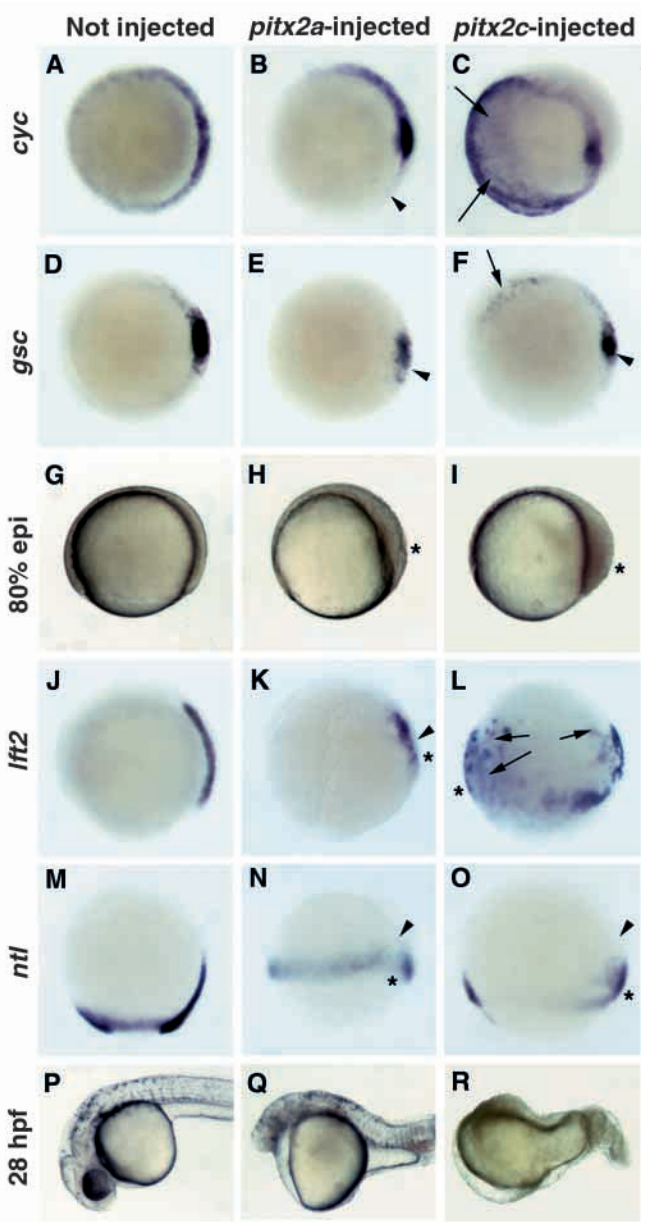


**Fig. 4.** Ectopic expression of *pitx2a* and *pitx2c* disrupts mesendoderm formation. Embryos were injected at the 1-2 cell stage with RNA. (A,D,G,J,M,P) Control or uninjected embryos. (B,E,H,K,N,Q) *pitx2a*-injected embryos. (C,F,I,L,O,R) *pitx2c*-injected embryos. (A-C) Expression of *cyc* at 40% epiboly; animal pole views with dorsal to the right. (B) *pitx2a*-injected embryo with arrowhead marking reduced expression of *cyc* in the margin. (C) *pitx2c*-injected embryo with arrows marking regions of increased *cyc* expression. (D-F) *gsc* expression at 40% epiboly; animal pole views with dorsal to the right. (E) A reduced number of cells with *gsc* expression in a *pitx2a*-injected embryo (arrowhead). (F) *pitx2c*-injected embryo with less *gsc* positive cells on the dorsal side (arrowhead) and ectopic activation of *gsc* expression in other marginal cells (arrow). (G-I) Lateral view of embryos at 80% epiboly. (G) Control embryo with dorsal to the right. (H,I) *pitx2a*- and *pitx2c*-injected embryos displaying slowed epiboly and excess cells at the blastoderm margin (asterisks). (J-L) Expression of *lft2* at 80% epiboly. (K,L) Asterisks mark an increased number of cells at the margin, arrowhead shows reduced expression, and arrows designate regions of ectopic expression of *lft2*. (M-O) Expression of *ntl/brachyury* at 80-90% epiboly. (M) Control embryo with dorsal to the right. (N,O) *pitx2a*- and *pitx2c*-injected embryos displaying slowed epiboly and a lack of dorsal accumulation of *ntl* (arrowheads). Asterisks mark an increased number of cells at the margin. (P-R) Lateral view of 28 hpf embryos with anterior to left.

**Table 1. The early expression of *pitx2c* is regulated by genes that control marginal mesendoderm and prechordal plate specification and differentiation**

| | Embryos lacking *pitx2c* expression | | | | | |
|---|---|---|---|---|---|---|
| | at 40-50% epiboly | | | at 80-90% epiboly | | |
| Mutant background | *n* | Marginal mesendoderm | Dorsal mesendoderm | *n* | Posterior prechordal plate | Polster |
| *cyc*$^{b229}$ | 146 | 0% | 0% | 70 | 31% | 31% |
| *cyc*$^{tf219}$ | n.d. | n.d. | n.d. | 30 | 23% | 23% |
| *igu* | 137 | 0% | 0% | 59 | 29%* | 29%* |
| *ntl* | 54‡ | 0% | 0% | n.d. | n.d. | n.d. |
| *oep* | 47 | 26% | 26% | 48 | 23% | 23% |
| *spt* | 132 | 0% | 29% | 51 | 31% | 0% |
| *sur* | 67 | 0% | 0% | 89 | 20% | 20%* |

Embryos from heterozygous intercrosses of the genotype indicated were analyzed at 40-50% or 80-90% epiboly for the mesendoderm and the prechordal plate expression of *pitx2*, respectively. Examples of altered expression are shown in Fig. 3. *n*, the total number of embryos analyzed; n.d., not determined.

*Expression of *pitx2* was reduced rather than absent in these mutants.

‡Embryos were scored at shield stage for *pitx2* expression.

To further examine the temporal onset of expression of each isoform, northern blot hybridizations were performed (Fig. 2B). In agreement with in situ hybridization, *pitx2c* was detected during dome stage while *pitx2a* was not observed until bud stage. Furthermore, *pitx2a* showed strong expression during heart looping stages (30-36 hpf) and at 48 hpf. The differential onset of *pitx2* isoform expression suggests that *pitx2c* plays an earlier role than *pitx2a* and that these isoforms have distinct roles during early anterior mesendoderm development.

### *spadetail* and *oep* regulate distinct *pitx2c* mesendoderm domains

To understand the regulation of *pitx2c* and its involvement in early mesendoderm development, the expression of *pitx2* was examined in mutant embryos defective in early mesendoderm development. In mutants in which *pitx2* expression was changed, the alterations occurred in approximately one quarter of the embryo population analyzed from intercrosses of identified heterozygotes (Table 1). Two mutants, *spadetail* (*spt*) and *oep*, displayed altered *pitx2* expression at 50% epiboly. *spt* corresponds to a member of the T-box class of transcription factors (Griffin et al., 1998), and mutant embryos display defects in specification of trunk and tail mesoderm but no obvious head defects (Ho and Kane, 1990). At 40% epiboly cells fated to become the shield in *spt* mutant embryos do not undergo compaction and exhibit defects in dorsal convergence (Warga and Nusslein-Volhard, 1998). In *spt* mutant embryos at 50% epiboly, *pitx2* expression was absent from superficial dorsal cells but still expressed in non-dorsal marginal cells (Fig. 3A,B).

At 50% epiboly, *pitx2* expression was completely absent in *oep* mutant embryos (Fig. 3C), which display strong defects in anterior mesendoderm specification. The zebrafish *oep* gene encodes a novel EGF-CFC transmembrane molecule that is implicated in mediating cell-cell signaling by the TGFβ family members Cyc and Sqt (Gritsman et al., 1999; Zhang et al., 1998). In contrast to the loss of *pitx2c* expression in *oep* mutants, *pitx2* expression was normal in *cyc* (*cyc*$^{b229}$) mutant embryos during these early stages (Table 1). This indicates the *oep*-dependent expression of *pitx2c* at late blastula stages does not occur solely through the *cyc* pathway; it might be regulated in conjunction with another member of the *nodal* family such as *sqt*. *pitx2* expression was also normal during late blastula stages in other mutant embryos defective in mesendoderm development and dorsal specification such as *no tail* (*ntl*), *iguana* (*igu*) and *schmalspur* (*sur*), suggesting the altered *pitx2c* expression in *spt* and *oep* is not merely due to alterations in cell fate. Together, these results implicate *spt* and *oep* in the specific regulation of both *pitx2c* expression and early mesendoderm development.

### Expression of *pitx2c* is disrupted in zebrafish prechordal plate mutant embryos

To examine the regulation of *pitx2c* during early prechordal plate development, *pitx2* expression was examined at 80-90% epiboly in *igu*, *spt*, *sur*, *cyc* and *oep* mutant embryos (Table 1). With the exception of *spt*, all these mutant embryos display varying degrees of cyclopia due to defects in the specification of ventral cell fates in the CNS (Brand et al., 1996). In *igu* mutant embryos, *pitx2* expression appeared in a narrower domain, indicating reduced prechordal plate mesendoderm (Fig. 3D,E). This suggests in addition to the strong CNS defects resulting in posterior cyclopia (Brand et al., 1996), *igu* mutant embryos also have reduced prechordal plate mesendoderm. In agreement with the early reduction in *pitx2* expression on the dorsal side of the embryo, *spt* mutant embryos displayed a loss of *pitx2* expression in the posterior domain of the prechordal plate, but maintained expression in the polster (Fig. 3F). In *sur* mutant embryos, which display posterior cyclopia similar to *igu* mutant embryos, *pitx2* expression was absent except for a small domain in the polster (Fig. 3G). In contrast to the normal expression of *pitx2* at 40% epiboly in *cyc* mutant embryos, *pitx2* expression was absent at 90% epiboly in *cyc* mutants except in a very small number of cells at the anterior end of its normal expression domain (Fig. 3H). This effect was observed with both a deletion (*cyc*$^{b229}$) and a point mutation (*cyc*$^{tf219}$) allele of *cyc* (Table 1). As in *oep* embryos at 50% epiboly, *pitx2* expression was also absent from the prechordal plate at 80% epiboly (Fig. 3I). In contrast, *ntl* mutant embryos, which display defects in notochord morphogenesis, showed no alteration of *pitx2* expression in the prechordal plate domain at shield stage (Table 1). Taken together, *pitx2c* expression is regulated either directly or indirectly in the prechordal plate mesendoderm by *igu*, *spt*, *sur*, *cyc* and *oep*.

**Table 2. *pitx2a* and *pitx2c* differentially regulate mesendoderm patterning**

| | Uninjected | *pitx2a*-injected | *pitx2c*-injected |
|---|---|---|---|
| Marker gene expression | | | |
| *cyc* | Normal | Reduced | Ectopic |
| | 96% (48) | 26% (27) | 75% (28) |
| *gsc* | Normal | Reduced | Reduced/ectopic |
| | 100% (189) | 36% (42) | 52% (42) |
| *lft2* | Normal | Reduced | Ectopic |
| | 100% (7) | 65% (17) | 68% (19) |
| *ntl* | Normal | Reduced | Reduced |
| | 100% (9) | 50% (18) | 75% (24) |
| Embryo phenotypes | | | |
| *n* | 457 | 150 | 89 |
| headless | 0% | 39%* | 63% |
| no notochord | 0% | 79% | 89% |

Embryos were scored at 30-40% epiboly for *cyc* and *gsc* and at 80% epiboly for expression of *ntl* and *lft2* by in situ hybridization. Examples of altered expression are displayed in Fig. 4.

Embryos were scored at 26-28 hpf for phenotype. *n*, total number of living embryos scored; numbers in parentheses are the number of embryos scored after in situ hybridization.

* 38 embryos were scored in an independent experiment.

## Pitx2a and Pitx2c differentially affect mesendoderm formation

*pitx2* isoforms have distinct expression patterns during early mesendoderm development. To test whether this corresponds to functional differences in the activity of *pitx2* isoforms, *pitx2a* and *pitx2c* were ectopically expressed in zebrafish embryos by injection of synthetic mRNA and hybridized with mesendodermal markers. *cyc* is expressed in the mesendoderm during late blastula stages and the embryonic midline during late zebrafish gastrulation (Rebagliati et al., 1998a; Sampath et al., 1998). At 40% epiboly, *cyc* was expressed in a ring of marginal cells and concentrated dorsally (Fig. 4A), similar to the expression of *pitx2c*. Ectopic expression of *pitx2a* downregulated *cyc* expression in marginal cells (Table 2, Fig. 4B). However, injection of *pitx2c* RNA upregulated *cyc* expression in both marginal and animal cap cells (Fig. 4C), suggesting that Pitx2a and Pitx2c have opposite effects on *cyc* expression. The ectopic activation of *cyc* by Pitx2c was also observed at 80% epiboly (data not shown).

Like *pitx2*, *goosecoid* (*gsc*) is a member of the *bicoid*-class of transcription factors and is expressed in dorsal marginal cells during late blastula stages in zebrafish embryos, corresponding to the future embryonic shield or organizer region (Fig. 4D; Stachel et al., 1993). During late gastrulation, *gsc* expression is restricted to the prechordal plate mesendoderm. *gsc* expression domains are conserved in other vertebrate species (Blum et al., 1992; Cho et al., 1991; Izpisua-Belmonte et al., 1993), and disruption of *gsc* in mice by homologous recombination results in prechordal plate and craniofacial defects (Belo et al., 1998; Rivera-Perez et al., 1995; Yamada et al., 1995). In *Xenopus*, ectopic *gsc* expression dorsalizes embryos and can induce a secondary embryonic axis (Cho et al., 1991). In embryos injected with *pitx2a* or *pitx2c* RNA, the number of *gsc* expressing cells on the dorsal side was reduced (Table 2, Fig. 4E,F), suggesting a reduction in dorsal development. However, unlike Pitx2a, Pitx2c produced ectopic patches of *gsc* expression in other marginal cells (Fig. 4F). This effect of ectopic Pitx2c on *gsc* correlates with the ectopic activation of *cyc* by Pitx2c (Fig. 4C).

Ectopic expression of either *pitx2a* or *pitx2c* disrupted gastrulation and led to an accumulation of cells at the blastoderm margin by late gastrulation (Fig. 4G-I). This phenotype was also associated with slowed epiboly relative to control embryos. To examine the nature of this perturbation, injected embryos were hybridized with *lft2*. At 80% epiboly, *lft2* is expressed in prechordal plate mesendoderm and floor plate precursors (Fig. 4J; Bisgrove et al., 1999). Injection of *pitx2a* and *pitx2c* RNA had differing effects on *lft2* expression (Table 2, Fig. 4K,L). In *pitx2a*-injected embryos, *lft2* expression was shortened along the anteroposterior axis and was not observed in cells at the margin (Fig. 4K). In contrast, *lft2* expression in *pitx2c*-injected embryos was observed in both the presumed midline and regions of cell accumulation at the blastoderm margin (Fig. 4L), indicating differences in the regulation of *lft2* by Pitx2a and Pitx2c may exist. Based on in situ hybridization analysis with *lft2* (Fig. 4J-L) and *cyc* (data not shown), the excess cells located at the blastoderm margin during gastrulation resulting from *pitx2a*- and *pitx2c*-injection were not necessarily coincident with the dorsal side of the embryo. Ectopic expression of either *pitx2* isoform may increase a cohesive cell behavior in groups of cells, possibly leading to a delay in epiboly and an increased number of cells that fail to migrate from the blastoderm margin. However, the fate of these cells may differ in embryos injected with *pitx2a* and *pitx2c*.

Injected embryos were further analyzed for the effects of *pitx2* isoforms on mesoderm patterning by hybridization with *ntl*. At 80-90% epiboly, *ntl* is expressed in two domains corresponding to the notochord on the dorsal side and to the mesoderm undergoing involution at the blastoderm margin (Fig. 4M; Schulte-Merker et al., 1992). In *pitx2a*- or *pitx2c*-injected embryos, which displayed slowed epiboly, *ntl* expression was severely reduced in the notochord domain compared to uninjected embryos at 80-90% epiboly (Fig. 4M-O). Additionally, the ring of *ntl* expression at the blastoderm margin was disrupted in many injected embryos, suggesting that mesoderm specification was disorganized in injected embryos. Correlating with disrupted cell movements and reduced *ntl* expression during gastrulation, many *pitx2a*- and *pitx2c*-injected embryos at 28 hpf displayed severe axis defects, including notochord loss and reduced dorsoanterior structures (Table 2, Fig. 4P-R). Consistent with *pitx2* isoforms being involved in distinct regulatory pathways, *pitx2c*-injected embryos displayed a higher proportion of embryos with reduced head development (Table 2).

## Pitx2a, Pitx2c and Gsc auto-regulation and cross-regulation

Analysis of injected embryos indicated distinct differences in the activities of Pitx2a and Pitx2c on mesendoderm marker gene expression. To further explore these differences, embryos injected with *pitx2a* or *pitx2c* were examined at late blastula stages for their ability to cross-regulate each other's expression. Additionally, the effects of ectopic *pitx2* expression were compared to the effects of ectopic *gsc* expression. Embryos injected with *pitx2a* displayed ectopic activation of *pitx2a* in both marginal and animal pole cells at 30% epiboly (Fig.

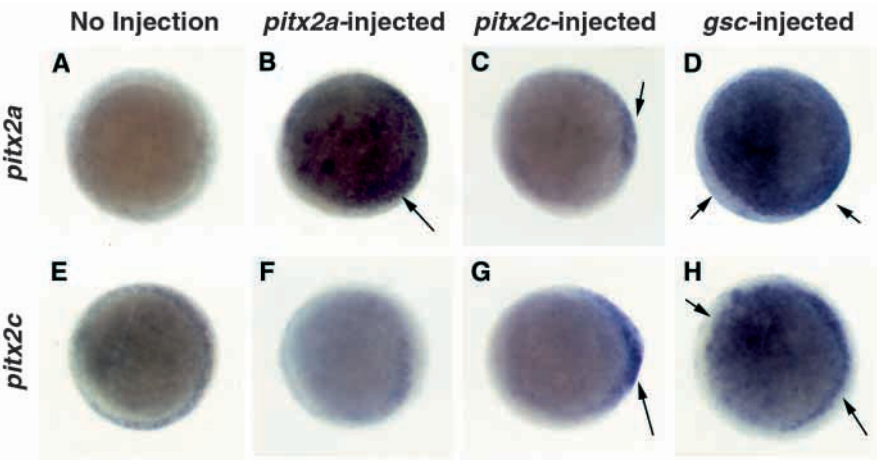


**Fig. 5.** Ectopic expression of *pitx2a, pitx2c* and *gsc* affects the expression of *pitx2a* and *pitx2c* at 30-40% epiboly. (A,E) Control embryos. (B,F) *pitx2a*-injected embryos. (C,G) *pitx2c*-injected embryos. (D,H) *gsc*-injected embryos. Embryos were analyzed for gene expression by in situ hybridization. (A-D) Expression of *pitx2a* mRNA (5′-UTR probe). (E-H) Expression of *pitx2c* mRNA (5′-UTR probe). Animal pole views are shown in all panels. Arrows designate regions of increased expression.

5A,B), suggesting a *pitx2a* autoregulatory loop. In contrast, *pitx2a*-injected embryos displayed no induction of *pitx2c* (Fig. 5E,F). *pitx2c* injected embryos displayed ectopic expression of *pitx2a* and itself, but only in the marginal zone and not in the animal pole cells (Fig. 5C,G). *gsc*-injected embryos displayed activation of *pitx2a* and *pitx2c* in both marginal and animal pole cells (Fig. 5D,H), suggesting Gsc positively regulates both *pitx2a* and *pitx2c* expression. Together with the early *pitx2c* and late *pitx2a* expression in the prechordal plate mesendoderm, we suggest that Gsc regulates *pitx2* isoforms during both early dorsal specification and later prechordal plate development.

### *pitx2a* and *pitx2c* are expressed in different asymmetric domains during the development of the diencephalon, gut and heart

At 22-25 somites of development, *pitx2* was expressed asymmetrically on the left side in the dorsal diencephalon and developing gut and heart fields (Fig. 6A). *pitx2* was also expressed at these stages in the trunk in presumed Rohon-Beard neurons (Fig. 6A), symmetrically at the base of the diencephalon (Fig. 6D), and in superficial cells on the yolk that likely contribute to the hatching gland (Fig. 6A). In situ hybridizations with isoform-specific probes displayed striking differences between *pitx2a* and *pitx2c* expression in asymmetric domains. *pitx2c*, but not *pitx2a*, was expressed asymmetrically in the dorsal diencephalon (Fig. 6A-F). The expression in the dorsal diencephalon most likely corresponds to the future habenulae nucleus, a structure known to exhibit both structural and functional differences between the left and right side in vertebrate organisms (Morgan, 1991). In the developing gut, only left-sided expression of *pitx2c* was detected (Fig. 6A-C,G-I).

Conversely, *pitx2a*, but not *pitx2c*, displayed left-sided, asymmetric expression in the developing heart field (Fig. 6A-C,J-L). This expression was strongest in the anterior regions of the heart field. In zebrafish, anterior regions of the heart field

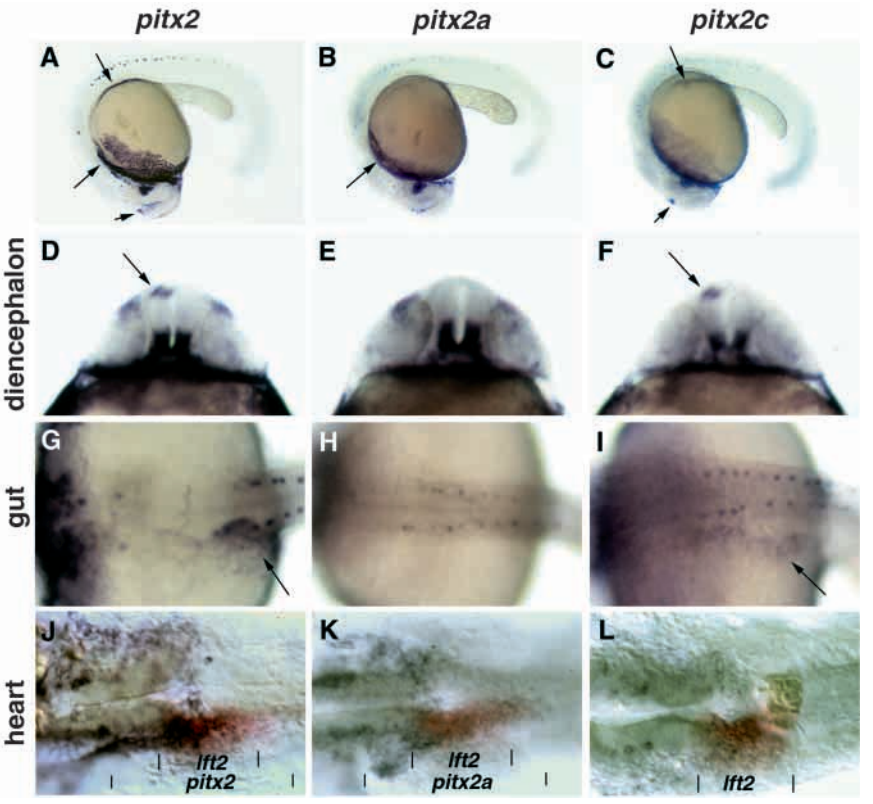


**Fig. 6.** *pitx2a* and *pitx2c* are differentially expressed during the asymmetric development of the diencephalon, gut and heart. In situ hybridizations at 22-25 somites of development using a full-length *pitx2* probe (A,D,G,J), a *pitx2a* isoform specific probe (B,E,H,K) and a *pitx2c* isoform specific probe (C,F,I,L). (A-C) Lateral views with arrows pointing to asymmetric expression in the dorsal diencephalon, the developing heart and gut from anterior to posterior. (D-F) Frontal views; arrows mark left-sided asymmetric expression in the dorsal diencephalon. Both isoforms were expressed symmetrically in the ventral diencephalon. (G-I) Dorsal views with arrows marking left-sided expression in the developing gut. (G-I) Double in situ hybridization with *pitx2* or *pitx2*-isoform specific probes (blue) and *lft2* (red) probes showing asymmetric expression of *pitx2* and *lft2* in the heart field.

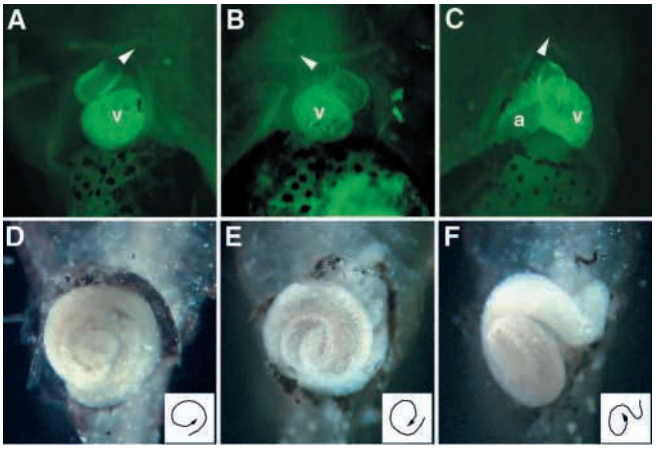


**Fig. 7.** Ectopic expression of either *pitx2a* or *pitx2c* affects heart and gut morphogenesis, including laterality, in *Xenopus* embryos. Embryos were injected at the 4-cell stage on the right side. (A) Normal heart morphology in an uninjected *Xenopus* embryo. (B) Reversed heart in an embryo injected with *pitx2a* DNA. (C) Malrotated heart with normal laterality in an embryo injected with *pitx2c* DNA. Embryos were fixed at stage 46 and processed for immunocytochemistry with an anti-cardiac troponin T antibody. Arrowheads indicate the direction of the heart outflow tract. a, atrium, v, ventricle. (D) Normal coiling of the gut in a uninjected embryo. Diagram depicts the counterclockwise coiling of the gut. (E) Reversed gut coil in an embryo injected with *pitx2a*. (F) Abnormal and reversed gut coiling in an embryo injected with *pitx2a*. Insets in E and F show improper initiation of coiling.

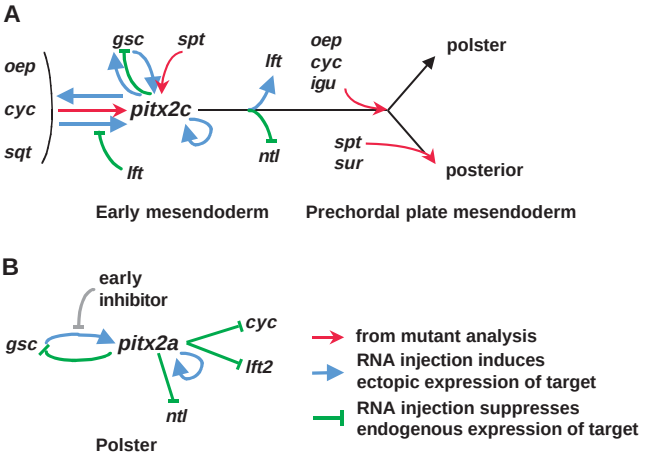


**Fig. 8.** Model of genetic interactions involving *pitx2* isoforms during early mesendoderm and prechordal plate development in zebrafish. Red arrows indicate associations deduced from analysis of mutant zebrafish embryos. Blue arrows show relationships where increased expression of *pitx2* isoforms induces ectopic expression of targets. Green lines represent negative regulation of endogenous expression as a result of ectopic expression. Black lines indicate decisions in cell fate. (A) Regulation of *pitx2c*. (B) Regulation of *pitx2a*. Antagonistic interactions between *cyc* and *lft* and *lft* regulation of *pitx2* were shown by Bisgrove et al. (1999). In addition to showing that *pitx2c* is reduced in *cyc* and *oep* mutants (this study; blue arrow), the regulation of *pitx2* by ectopic expression of *nodal* (*Xnr1* in *Xenopus* or *cyc* in fish) was shown by Campione et al. (1999) (red arrow).

include the future atrium (Stainier and Fishman, 1992), opposite to the orientation in other vertebrate species. As the yolk is absorbed, the heart comes to lie in its normal orientation with the atrium to the posterior. To further mark different regions of the heart primordia, embryos were hybridized with *lft2* (red), which also has left-sided expression in the heart field during late somite stages (Fig. 6J-L) (Bisgrove et al., 1999). The strongest asymmetric expression of *pitx2a* in the developing heart overlapped with *lft2* in the anterior domain of *lft2* asymmetric expression. Weaker expression of *pitx2a* was also observed in left/lateral regions of the *lft2* domain. The absence of a complete overlap of *pitx2a* and *lft2* domains suggests that *pitx2a* and *lft2* may have different zones of activity in the development of heart asymmetry. Together, these results indicate that different organ primordia utilize distinct *pitx2* isoforms during left-right development.

## Ectopic expression of *pitx2* isoforms in *Xenopus* embryos affects heart and gut laterality

*pitx2a* was expressed asymmetrically in the heart field, while *pitx2c* transcripts were found in both the left diencephalon and developing gut (Fig. 6). To examine the potentially distinct roles of *pitx2* isoforms in laterality and heart and gut morphogenesis, targeted left or right expression of *pitx2a* or *pitx2c* was carried out in *Xenopus* embryos. Due to the indeterminate orientations of the early cleavage planes (Helde et al., 1994), injections targeted to the prospective left or right sides are not as readily feasible in zebrafish. *Xenopus* allows targeted injection of molecules into either the left or right sides and serves as a superb assay system for candidate genes in left-right development (Hyatt and Yost, 1998; Ramsdell and Yost, 1999). Zebrafish *pitx2a* or *pitx2c* DNA expression constructs were injected into the ventral left or ventral right blastomere of 4-cell *Xenopus* embryos, and the effects of ectopic expression on heart and gut morphogenesis were observed on day 5 of development (stage 46).

Normally at stage 46, the heart outflow tract, the conus truncus, extends anteriorly from the right side of the ventricle and loops to the left of the embryo, as shown in uninjected control embryos stained with anti-cardiac troponin T (Fig. 7A). Injection of either *pitx2* isoform on the left or right side of the embryo was able to reverse heart laterality (Table 3), with the conus extending from the anterior-left side of the ventricle and looping to the right (Fig. 7B). However, right-sided injection of *pitx2a*, the isoform expressed asymmetrically in the developing zebrafish heart, produced approximately double the number of heart reversals as compared to *pitx2c* injections ($P<0.1$). Furthermore, injection of either isoform into either side sometimes resulted in malrotation of the heart, with both the atrium and ventricle displaced lateral to their normal position (Table 3, Fig. 7C). This effect was observed at a greater frequency in the right-side injected specimens than in the left-injected, and it did not appear to correlate with the laterality phenotype since the incidence of reversals among the malrotated hearts was no higher than that observed among all experimental hearts (data not shown). Although both Pitx2a and Pitx2c altered heart morphogenesis, their competence to affect cardiac laterality was distinct.

The laterality and morphogenesis of the gut was also affected by injection of *pitx2a* and *pitx2c* (Fig. 7D-F, Table 3). At stage 46 in *Xenopus*, the gut extends caudally from the

**Table 3. Ectopic expression of either *pitx2a* or *pitx2c* affects heart and gut laterality in Xenopus embryos**

| | | Heart | | Gut | | |
|---|---|---|---|---|---|---|
| Injection DNA | *n* | Reversal | Malrotation | Reversal | Abnormal | Reversed hearts and guts* |
| Uninjected | 376 | 2% | 1% | 3% | 4% | 29% (7) |
| *pitx2a* | | | | | | |
| right | 84 | 28% | 17% | 38% | 42% | 76% (21) |
| left | 84 | 16% | 8% | 8% | 19% | 36% (11) |
| *pitx2c* | | | | | | |
| right | 74 | 16% | 24% | 35% | 41% | 70% (10) |
| left | 79 | 13% | 6% | 9% | 20% | 50% (10) |

*pitx2a* and *pitx2c* DNAs were injected into the left or right side of 4-cell *Xenopus* embryos, and these specimens were scored for heart and gut laterality at 5 days of development (stage 46). Scoring of heart laterality was based upon the positioning and looping of the outflow tract. The phenotype termed 'heart malrotation' can be visualized in Fig. 7C. Gut reversal denotes that the major gut coil was on the right side of the abdominal cavity (Fig. 7E). Abnormal gut refers to specimens in which the gut morphology, particularly the pattern of coiling, was aberrant in comparison to uninjected controls (Fig. 7F).

*Percentage of embryos with reversed hearts that also displayed reversed guts. The number in parentheses is the total number of embryos with reversed hearts that could be scored for gut laterality.

esophagus along the right lateral wall of the abdominal cavity, traverses the cavity posteriorly, and forms a large counterclockwise coil on the left side of the embryo (Fig. 7D; Chalmers and Slack, 1998). Injection of either *pitx2* isoform on either side of the embryo was able to reverse gut morphology, resulting in the major intestinal coil being located on the right side of the abdominal cavity (Table 3, Fig. 7E). In addition, many of the injected embryos displayed disorganized coiling of the gut (Fig. 7F). Both dysmorphologies were more frequently observed with right-sided injections. In addition, a higher proportion of embryos had both reversed hearts and reversed guts when *pitx2* isoforms were injected on the right rather than on the left side of the embryo (Table 3). These results further demonstrate the ability of *pitx2* isoforms to affect morphogenesis and laterality of the internal organs.

## DISCUSSION

We have isolated two isoforms of *pitx2* from zebrafish, which show strong conservation to *pitx2a* and *pitx2c* from other vertebrate species. *pitx2c* is expressed during early mesendoderm specification and throughout prechordal plate mesendoderm during gastrulation. In contrast, *pitx2a* expression is first expressed after gastrulation in only the anterior prechordal plate (polster). Analysis of expression patterns in zebrafish embryos indicates that *pitx2* isoforms are under distinct regulation, both spatially and temporally, and may have distinct functions during mesendoderm development. Fig. 8 summarizes the genetic pathways identified by mutant analysis and ectopic expression experiments.

### *pitx2c* expression in the early mesendoderm is regulated by TGFβ signaling cascades

During late blastula stages, *pitx2c* is expressed at the blastoderm margin in cells fated to become both mesoderm and endoderm (Warga and Nusslein-Volhard, 1999). As development proceeds through gastrulation, cells expressing *pitx2c* have characteristics of early endoderm (Fig. 2 and data not shown), including a flattened appearance and a deep location in the blastoderm next to the yolk cell (Warga and Nusslein-Volhard, 1999). Specification of the early endoderm has been shown to require TGFβ signaling, whereas mesoderm specification is thought to require both TGFβ and Fibroblast Growth Factor (FGF) signaling pathways (Rodaway et al., 1999). Consistent with a requirement for TGFβ signaling, the expression pattern of *pitx2c* during late blastula stages is similar to those of members of the TGFβ family, including *cyc* (Rebagliati et al., 1998a), *sqt* (Erter et al., 1998; Rebagliati et al., 1998a), *lft1* (Bisgrove et al., 1999; Thisse and Thisse, 1999), *lft2* (Bisgrove et al., 1999) and a modulator of *nodal* signaling, *oep* (Gritsman et al., 1999).

Several lines of evidence show that *pitx2c* is regulated by TGFβ signaling pathways (Fig. 8). The analysis of *pitx2c* expression in *oep* mutants indicates that *oep* acts as an upstream regulator of *pitx2c* expression. Homozygous mutant *oep* embryos display severe defects in mesendoderm formation (Schier et al., 1996; Solnica-Krezel et al., 1996). Oep, an EGF-CFC transmembrane protein (Zhang et al., 1998), may directly modulate Cyc and Sqt signaling (Gritsman et al., 1999), and together with Cyc and Sqt could positively regulate *pitx2c* expression. Contrary to this hypothesis, *pitx2c* expression is normal during late blastula stages in *cyc* mutant embryos (Table 1). This probably reflects an overlapping function of *cyc* and *sqt* during this time (Erter et al., 1998; Rebagliati et al., 1998a). In support of a partially redundant function, *cyc*/*sqt* double mutant embryos have a more severe reduction in mesendoderm, similar to *oep* mutant embryos (Gritsman et al., 1999). Furthermore, *pitx2c* expression is absent later in *cyc* mutants, after *sqt* expression is no longer present. In *Xenopus*, ectopic expression of the *nodal*-related gene *Xnr1*, a homolog of *cyc*, dramatically upregulates *pitx2* in animal cap assays (Campione et al., 1999). In both mice and zebrafish, *lft*, another member of the TGFβ family, antagonizes the activity of *cyc* (*nodal*) signaling (Bisgrove et al., 1999; Meno et al., 1999). Ectopic expression of *lft1* in zebrafish eliminates the expression of *pitx2* at 50% epiboly (Bisgrove et al., 1999). Together these data suggest that *pitx2* expression in the mesendoderm is regulated by a balance between the activities of the mutually antagonistic TGFβ signaling molecules, Cyc and Lft.

### Pitx2 isoforms and Gsc have opposing activities

During early dorsoventral patterning of the mesendoderm, Pitx2 and Gsc, both members of the *bicoid*-homeobox class of transcription factors, have opposing activities and regulate the

expression of one another. *gsc* expression leads to dorsal development (data not shown) and upregulates *pitx2a* and *pitx2c* at late blastula stages. This agrees with studies using *Xenopus* animal cap assays where expression of *gsc* upregulated *pitx2* (Campione et al., 1999), although in these studies different *pitx2* isoforms were not distinguished. Given its activity to regulate *pitx2a* and *pitx2c* expression during late blastula stages, Gsc may normally function at different stages of prechordal plate development to affect the expression of both *pitx2* isoforms. Early in dorsal mesendoderm formation and later throughout the prechordal plate, Gsc may positively upregulate *pitx2c* (Fig. 8A). *pitx2a* is not expressed until after gastrulation and could be repressed by an unknown inhibitor (Fig. 8B). Later, when the inhibition is absent in the polster, Gsc could positively regulate the transcription of *pitx2a* (Fig. 8B). Our data also shows Pitx2a and Pitx2c negatively affect *gsc* expression and promote a ventralized phenotype. The opposing activities of Gsc and Pitx2 isoforms during dorsal mesendoderm development suggest that a balance between the activities of Gsc, Pitx2a and Pitx2c is required for the anterior and posterior identity of the prechordal plate mesendoderm at multiple stages of development.

### Pitx2 isoforms regulate cell behavior in the early mesendoderm

Both Pitx2a and Pitx2c regulate changes in cell behavior. Ectopic expression of either isoform disrupts gastrulation and leads to excess cells at the blastoderm margin (Fig. 4). This phenotype could be a result of increased cellular adhesion in cells overexpressing Pitx2 isoforms. Ectopic expression by RNA injection at the 1- to 2-cell stage leads to mosaic expression in zebrafish, with patches of cells expressing the RNA in random locations within the blastoderm. In *pitx2*-injected embryos, the random location of the accumulation of cells at the blastoderm margin with respect to the dorsoventral axis is consistent with the mosaic nature of ectopic expression experiments. This accumulation during gastrulation most likely reflects a cell-autonomous effect of expressing Pitx2a or Pitx2c in these cells, possibly by causing cells to interact with one another rather than migrate to their normal positions.

During late blastula stages in zebrafish, presumptive mesendodermal cells in the dorsal marginal zone flatten and maximize cellular contact with one another (Warga and Nusslein-Volhard, 1999), likely occurring through an increase in cellular adhesion among these cells. This cohesive behavior is disrupted in *spt* mutant embryos (Warga and Nusslein-Volhard, 1998). In addition, *paraxial protocadherin* (*papc*), encoding a cell adhesion molecule, is a downstream target of *spt* (Yamamoto et al., 1998). Consistent with a role for Pitx2 isoforms in mediating changes in cellular adhesion, our data show that *spt* regulates the early dorsal-marginal expression of *pitx2c* (Fig. 3). *pitx2* may have an intermediate role between *spt* and changes in cell behavior mediated through *papc* and other cell adhesion molecules. Similar alterations in cell behavior could elicit morphological asymmetries during left-right development.

The observation that ectopic Pitx2a and Pitx2c both led to the accumulation of cells during gastrulation implies that these isoforms have some similar regulatory functions (Fig. 4). The phenotypes observed at 28 hpf after injection of either *pitx2a* or *pitx2c* RNA also support this conclusion, and the loss of notochord and anterior structures could be a direct result of a failure of axial mesendodermal cells to migrate to their normal positions during gastrulation. However, Pitx2a and Pitx2c also appear to have distinct functions in regulating *cyc*, *lft2* and *gsc* (Fig. 4). The observation of both similar and opposing effects of ectopic Pitx2a and Pitx2c suggests that the ability of *pitx2* isoforms to differentially affect gene transcription may be dependent on the context of the promoters in these target genes.

### Maintenance of prechordal plate expression of *pitx2c* is regulated by *sur, spt, cyc* and *oep*

The maintenance of *pitx2c* expression during late gastrulation by *sur* and *spt* defines anterior and posterior domains in the prechordal plate mesendoderm (Figs 3, 8A). The absence of *pitx2c* expression during gastrulation in the posterior prechordal plate in *sur* mutant embryos correlates with the later observation of posterior cyclopia (Brand et al., 1996) and indicates that *pitx2c* expression is regulated differently in the anterior versus the posterior regions of the prechordal plate. Similar to *sur* mutant embryos, *spt* mutant embryos also lack *pitx2c* expression in the posterior prechordal plate. In contrast, *spt* mutants form normal notochord and head structures (Warga and Nusslein-Volhard, 1998), suggesting that *spt* and *pitx2c* expression in the posterior prechordal plate are not required for either head or midline development. *spt* and *sur* mutant embryos also have defects of the left-right patterning of the heart (Chen et al., 1997). The loss of *pitx2c* expression in the posterior prechordal plate of *spt* and *sur* mutant embryos may relate to a specific role for *pitx2c* and the posterior prechordal plate in left-right axis determination or maintenance.

During late gastrulation, maintenance of *pitx2c* expression in the polster and posterior prechordal plate requires the activities of *cyc* and *oep* (Figs 3, 8A). The alteration of *pitx2c* expression in *cyc* and *oep* mutants could reflect a secondary effect, due to either an absence of cells in the prechordal plate mesendoderm or a change in cell fate. In *cyc* mutants at least some cells maintain a prechordal plate identity; both *gsc* and *forkhead-domain 2* (*fkd2*) expression are present although reduced at 90% epiboly (Thisse et al., 1994; Warga and Nusslein-Volhard, 1999). The almost complete lack of *pitx2c* expression in *cyc* mutant embryos at 90% epiboly suggests that *pitx2c* expression is more directly regulated by Cyc signaling than *gsc* or *fkd2* expression and may be a proximal downstream target. While *oep* mutants have a severe reduction in anterior mesendoderm, which may account for the loss of *pitx2c* expression in this mutant at late gastrula stages, the earlier loss of *pitx2c* expression in *oep* mutants suggests more direct regulation of *pitx2c* by Oep. Based on our mutant analysis, we suggest that Cyc and Oep signaling function together in the regulation of *pitx2c* expression.

### Pitx2 isoforms have different activities and regulate laterality

The *pitx2c* isoform acts in a feedback loop with *cyc* and *lft2*. In contrast to Pitx2a, ectopic Pitx2c can upregulate *cyc* and *lft2* expression during mesendoderm development (Figs 4, 8). Differences also exist between the ability of Pitx2a and Pitx2c to regulate each other and *gsc* (Figs 4, 5). Thus, *pitx2a* and *pitx2c* have divergent activities, which may be reflected in their different expression domains during both mesendoderm and left-right development.

The distinct patterns of *pitx2* isoform expression during asymmetric organ development imply the left-sided identity of different organ primordia is regulated by distinct mechanisms. In early zebrafish embryos, a balance between Cyc and Lft activities appears to regulate the expression of *pitx2c*, and *pitx2c* itself can feed back to induce both *cyc* and *lft2* expression. A similar mechanism may regulate *pitx2c* expression in the dorsal diencephalon. *lft1* (Bisgrove et al., 1999; Thisse and Thisse, 1999), *lft2* (Bisgrove et al., 1999) and *cyc* (Rebagliati et al., 1998a) are also expressed at late somite stages in the left dorsal diencephalon and, together with *pitx2c*, may maintain each others' expression in this tissue. However, in the heart and gut primordia a different mechanism exists to induce *pitx2a* and *pitx2c*.

*pitx2a* asymmetric expression does not entirely overlap with *lft2* in the left heart primordium (Fig. 6), while *cyc* appears to be broadly expressed in the left lateral plate mesoderm (Rebagliati et al., 1998a), suggesting that different mechanisms may regulate *lft2*, *cyc* and *pitx2* isoforms on the left side of the embryo. Furthermore, in early embryos, ectopic Pitx2a does not positively regulate either *lft2* or *cyc* expression and may function distinctly from Cyc and Lft2 later in the heart field. While both *pitx2* isoforms were equivalent in their ability to affect laterality in the gut in *Xenopus* embryos, Pitx2a had a stronger effect on heart asymmetry than Pitx2c, correlating with the normal expression of *pitx2a* in the zebrafish heart field. In addition, *lft1* and *lft2* are not expressed in the presumptive gut (Bisgrove et al., 1999), while *pitx2c* (this paper) and *cyc* (Rebagliati et al., 1998a) expression appear to overlap in this region. Based on their expression domains and our results in early zebrafish embryos, we hypothesize a regulatory loop between *cyc* and *pitx2c* in the gut primordium.

Our results indicate that overexpression of both Pitx2a and Pitx2c by DNA injection can perturb laterality in the developing heart and gut in *Xenopus*. However, Pitx2a has a stronger effect on the heart than Pitx2c (Table 3). Similar laterality phenotypes in *Xenopus* were previously observed as a result of targeted injection of *Xenopus Pitx2* RNA on the right side of the embryo (Campione et al., 1999; Ryan et al., 1998). However, these reports did not distinguish between different *pitx2* isoforms. Additionally, we found that *pitx2* isoforms affect left-right development when injected on either the left or right side of the embryo. As expected, right-sided injections, which would place *pitx2* expression ectopically on the right side of the embryo during asymmetric organ development, resulted in a higher frequency of laterality disturbances than left side injections, which serve to overexpress *pitx2* on its normal side of expression. Ectopic expression of *pitx2* on the right side or increased expression of *pitx2* on the left side may also affect left-right development at an earlier developmental stage. Our observation that *pitx2* isoforms can alter the expression of other genes implicated in earlier steps of left-right development in zebrafish, including *cyc* (Chen et al., 1996) and *ntl* (Danos and Yost, 1996), was surprising. These early disturbances may account for a certain frequency of *Xenopus* embryos exhibiting perturbed left-right morphogenesis following unilateral *pitx2* injection.

Ectopic expression of either *pitx2* isoform during early zebrafish development disrupted cell movements during gastrulation, possibly resulting from increased cell adhesion among blastomeres. This raises the intriguing possibility that *pitx2* isoforms may function during asymmetric organ development in a similar manner. Although the cellular mechanisms that are downstream of *pitx2* are unknown, *pitx2* isoforms may function in laterality decisions by regulating cell adhesion and cell migration differences between the left and right sides of organ primordia.

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