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# Cloning and Expression Pattern of Chicken *Pitx2:* A New Component in the SHH Signaling Pathway Controlling Embryonic Heart Looping

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**Asymmetry along the left-right axis of the embryo is a vital feature of vertebrate embryogenesis. In this study, we report the isolation and characterization of a *bicoid*-related homeobox gene, *cPitx2,* which displays left-right asymmetric expression during early chick embryogenesis. Asymmetric expression of *cPitx2* is first detected at stage 7 and is restricted to mesodermal tissues on the left side of the embryo including the left sided lateral mesoderm, the left sided precardiac mesoderm, and the left half epimyocardium of the primitive heart. *cPitx2* is also detected in the presumptive blood islands and endothelia of the embryonic blood vessels. Implantation of Sonic hedgehog (SHH) protein soaked beads on the right side of embryos induced ectopic *cPitx2* expression on that side. Based on these observations, we suggest that *cPitx2* is a component in SHH signaling pathway and plays a role in determining left-right asymmetry and in vasculogenesis during avian embryogenesis.** 

***Key Words: cPitx2,* chick embryo, left-right asymmetry, heart looping.**

During development, embryos portray complex patterns along multiple axes which include, anterior-posterior, dorsal-ventral, and left-right. While specification of both anterio-posterior and dorso-ventral axial asymmetries have been studied in some detail, the molecular mechanisms underlying left-right asymmetry have just begun to be elucidated (1, 2). In the developing chick embryo, orientation of the blastoderm relative to the yolk determines the dorso-ventral axis. Anterio-posterior axial determination follows with the formation of Koller's sickle and the primitive streak at the future posterior end of the embryo. It is not until these two axes are in place that the third axis, the left-right axis, is determined.

The first morphological indication of asymmetry between the left and right sides of the developing embryo appears during organogenesis of the heart. The avian heart is derived from two 'fields' of precardiac mesoderm which reside in the lateral mesoderm on either side of the primitive streak. Two separate heart tubes form as the precardiac mesoderm migrate towards the anterior, and then come together and fuse at the midline to form a single heart tube. This primitive heart tube then undergoes a dextral looping such that the tube twists to the embryo's right side, thus presenting the first morphological sign of left-right asymmetry. The timing of left-right asymmetry specification has been investigated by transplantation experiments. Studies by Hoyle et al. (3) suggested that in the developing chick, heart looping is controlled by the lateral plate mesoderm and that intrinsic changes influencing the direction of heart looping have occurred in the precardiac mesoderm during gastrulation. Dextral looping of the heart is a feature which has been conserved among all vertebrate species, suggesting the existence of an evolutionary conserved mechanism for this critical step in cardiac morphogenesis.

Recent studies have provided insight into the molecular basis for the rightward looping of the heart tube and a number of genes, shown to control the direction of heart looping, have been identified in the mouse (4-6), frog (7-9), chick (10-13) and fish (14). In the chick embryo these genes, which include *Sonic hedgehog (Shh), nodal, activin-βB,* activin receptor IIa (*cActRIIa*) and the *Snail* related gene (*cSnR*), exhibit a left-right asymmetric expression prior to the overt morphological asymmetry (i.e., prior to cardiac looping). The

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first of these genes to show asymmetric expression is *activin-βB*, which at stage $3^+$ (15) is detected in the right side of Hensen's node (11). *Activin-βB* then induces *cAct-RIIa* expression, also in the right side of the node. Prior to the expression of *cAct-RIIa*, *Shh* is expressed bilaterally throughout the node. However, at precisely the same time (stage 5) when *cAct-RIIa* is expressed, *Shh* expression becomes restricted to the left side of the node, suggesting that endogenous *activin-βB* is responsible for setting up the asymmetric expression of *Shh* (11). This hypothesis is supported by studies showing that the ectopic expression of *activin* on the left side of the node eliminates the endogenous *Shh* expression domain on the left side of the node (11). At stage 6, *Shh* is believed to induce the expression of *nodal*, a member of the TGF-β superfamily, in a small domain of cells just lateral to the node (10). The expression domain of *nodal* subsequently expands but remains restricted to the left sided lateral mesoderm (11). Concomitant with *nodal* expression on the left side of the embryo, *cSnR* expression becomes evident in the right sided lateral mesoderm (12). The ectopic expression of *Shh* on the right side of the node induces ectopic *nodal* expression but inhibits *cSnR* expression in the right sided lateral mesoderm, which results in randomization of heart looping (10, 12). On the other hand, randomization of heart looping can also be achieved by application of activin protein to the left side of node, which represses the expression of *Shh* and *nodal* and activates *cSnR* expression in the left sided lateral mesoderm (10-12). Based on these observations, it was proposed that left-right asymmetry is determined by a series of gene interactions, in which *activin* expression in the right side of the node induces *cAct-RIIa* in the same region and restricts *Shh* expression to the left side of the node. *Shh* further induces *nodal* expression but inhibits *cSnR* expression in the left sided lateral mesoderm. Therefore, the expression of *nodal* on the left side and *cSnR* on the right side of the embryo determines left-right asymmetry well before heart formation and heart looping (1, 10-13).

In this study we have cloned the chick homologue of *Pitx2*, named *cPitx2*, and determined that like *nodal*, *cPitx2* exhibits asymmetrical left-right expression prior to and during heart looping in developing chick embryo. *Pitx2* is a member of *Pitx/Rieg* family of homeobox containing genes. The *Pitx/Rieg* family of genes are closely related to the *bicoid* gene of *Drosophila* which has been shown to be important for body plan specification (16, 17). Several other members of this gene family have been recently identified (18-22). Mutations in *PITX2* (also known as *RIEG*) in humans have been shown to be responsible for the autosomal-dominant disorder, *Rieger* Syndrome (16). In the developing mouse embryo, *Pitx2* expression is detected in Rathke's pouch, periocular mesenchyme, limb mesenchyme, vitelline vessels, brain and dental epithelium (16, 17, 23). However, its precise function during embryogenesis is still unclear. Here we present evidence that *cPitx2* acts downstream from SHH signaling pathway, and that it may play a role in the determination of left-right asymmetry and in vascular formation during early embryogenesis.

## MATERIALS AND METHODS

*Cloning of cPitx2 cDNA.* To isolate the chicken *Pitx2* homeobox gene, a cDNA library prepared from day 10 chicken brain was screened with a mouse *Pitx2* cDNA probe (16) under low stringency conditions. Briefly, one million phage plaques were plated and hybridized with a mouse *Pitx2* probe in 6X SSPE, 5X Denhardts, 50mM Phosphate, 0.1% SDS, 10mM $Na_4P_2O_7$, 200μg/ml salmon sperm DNA at 60°C. Membranes were then washed three times for 30 minutes each with 4X SSPE, 0.2% SDS at 50°C before exposing to X-ray film.

*Whole-mount in situ hybridization.* For in situ hybridizations, the *cPitx2* containing plasmid DNA was linearized with *BamH*I and a 2-Kb antisense riboprobe was generated using T7 RNA polymerase in the presence of digoxygenin labeled UTP as directed by the manufacturer (Boehringer Mannheim Biochemicals). Probe size and yield were compared to RNA standard by agarose gel electrophoresis.

Samples were fixed in "M" buffer (3.7% formaldehyde, 100 mM MOPS, pH7.4, 2 mM EGTA, 1 mM $MgSO_4$) at 4°C overnight followed by bleaching with 10% $H_2O_2$ in "M" buffer at room temperature. Whole-mount in situ hybridization was performed as described previously (24). Briefly, the probe was hydrolyzed to approximately 600-bp by incubation with hydrolysis buffer at 60°C for 40 minutes prior to use. Embryos were treated with proteinase K treatment at 1 μg/ml for 5 minutes at room temperature. Signals were visualized using NBT/BCIP (Boehringer Mannheim). The color reaction was stopped by washing the samples with PBS followed by re-fixing with "M" buffer for 30 minutes. Samples were washed again with PBS and dehydrated in methanol prior to photography and sectioning. Fixed whole-mount samples were cleared with xylene followed by embedding in paraffin wax. Embryos were sectioned at 15 μm and mounted with Permount.

*Bead implantation.* SHH protein was prepared from a *Shh* expression vector containing sequences encoding six histidine residues upstream to the mouse *Shh*-coding sequences (amino acids 25-198). The SHH protein was induced in bacteria and purified as previously described (25). The inductive activity of the recombinant SHH protein was examined and confirmed by its ability to induce digit duplication in chick wing buds where beads soaked with SHH recombinant protein were implanted to the anterior margin of a host wing bud (data not shown). For experiments reported here, affi-Gel blue agarose beads (from Bio-Rad) were washed with PBS prior to incubating with 1.5 mg/ml SHH protein in 37°C water bath for 30 minutes. White heparin beads (Sigma) were chosen for controls for easy distinction, and were soaked with BSA under the same conditions. All protein-soaked beads were stored at 4°C and used within one week.

Bead implantation experiments were performed on stage 4 or stage 5 chick embryos both in ovo (26) and in vitro using New culture method (27), as described. In these assays, SHH or BSA proteins soaked beads were implanted on the left or right side of each embryo at the level of Hensen's node. Bead implanted embryos were allowed to develop to stage 11. Only embryos surviving the surgery were harvested and processed for whole mount in situ hybridization.

## RESULTS

### *Cloning of cPitx2*

*cPitx2* was cloned from a chicken brain library using a mouse *Pitx2* cDNA as probe (16). Under low strin-

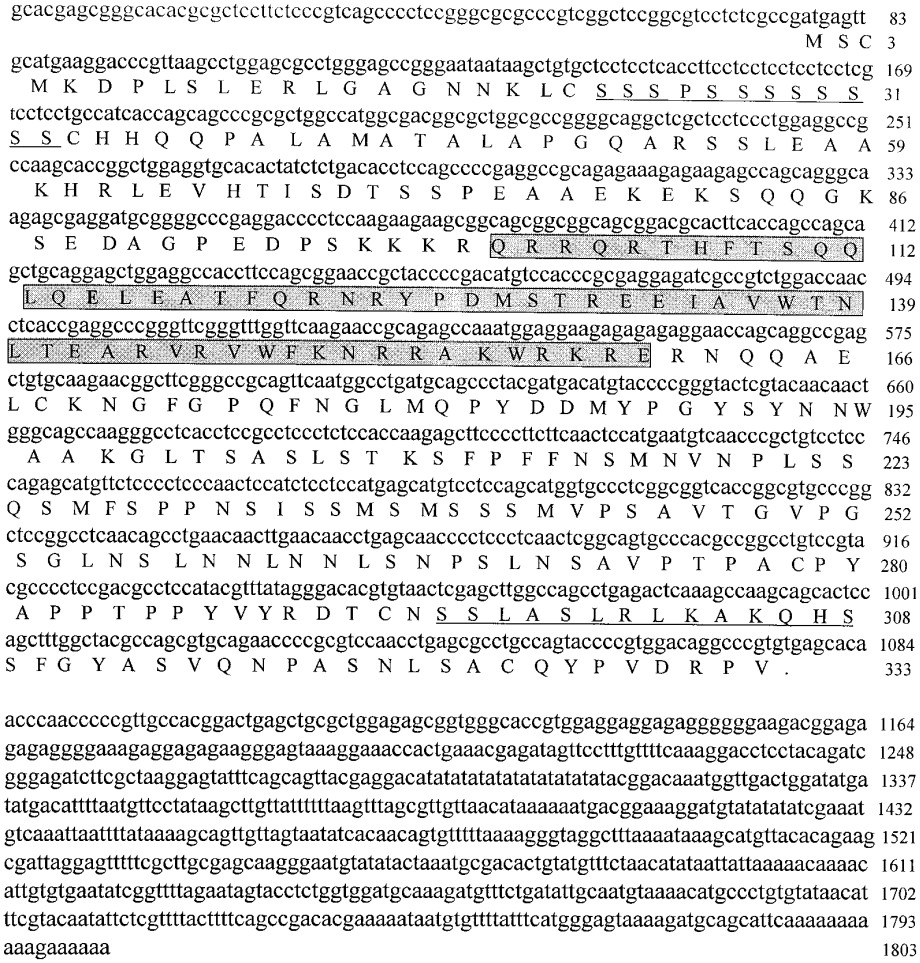



**FIG. 1.** Nucleotide and translated amino acid sequences of *cPitx2*. The homeobox is boxed and shaded, and the amino acid at position 15 of the first helix of the homeodomain, where cPitx2 and mouse Pitx2 differ from human RIEG (Q to E), is shown in bold. The stretch of serine residues at the N-terminus and the conserved 14 amino acid motif at the C-terminal end are underlined.

gency hybridization conditions, 25 independent clones were identified from the $1 \times 10^6$ clones examined. These clones were sequenced in both direction and analyzed for homology with known sequences deposited in genetic databases. From the 25 clones, 9 showed sequence homology to the mammalian *Pitx2* gene. Overlapping clones were compiled to construct a full length chick *cPitx2* cDNA contig of 1803bp (Fig. 1). The first methionine associated with the longest open reading frame was found at position 77 of the cDNA resulting in a 333 amino acid protein containing a putative homeodomain (codon 101 to 160).

At the nucleotide level, *cPitx2* is 76% identical to the mouse *Pitx2* and 77% identical to the human *RIEG*. At the protein level, cPitx2 differs from both mouse Pitx2 and human RIEG primarily in the N-terminal, 5′ to the homeodomain, and are almost completely similar throughout the rest of the protein including the homeodomain and C-terminal end with only a few amino acid changes between them. In the homeodomain, cPitx2 shares 100% similarity to the mouse Pitx2, and 98% similarity the human RIEG resulting from a single amino acid change at position 15 of the first helix (Q to E). The most characteristic aspect of cPitx2 is a stretch of serine residues in the region 5′ to the homeodomain. Similar stretches of repeated serine residues have also been found in other transcription factors, such as the *Nkx-6.1* homeobox gene (28). The function of this serine repeat is currently unknown. However, it may play a role in protein phosphorylation or transactivation of gene expression. cPitx2 also maintains the conserved fourteen amino acid motif which is common among the members of *Pitx/RIEG* family and some other selected homeodomain-containing proteins (22). Together these data indicate that *cPitx2* is the chicken homologue of the mammalian *Pitx2/Rieg* gene.

### *Expression of cPitx2 during Early Embryogenesis*

The expression pattern of *cPitx2* was analyzed by whole mount in situ hybridization on chicken embryos

ranging from stage 4 (early gastrulation) to stage 22. Asymmetric expression of *cPitx2* was first detected in stage 7 (Fig. 2a) embryos as a small patch, only on the left sided lateral mesoderm, just lateral and anterior to the node. Expression was also detected in the area opaca vasculosa and head mesenchyme with symmetrical pattern at this stage (Fig. 2a and data not shown). By stage 8 this patch of asymmetric expression extends anteriorly and posteriorly and was restricted to the left sided lateral plate mesoderm, as indicated by sections from the whole mount in situ embryos (Fig. 2b,e). This asymmetric expression of *cPitx2* remains in the left sided lateral mesoderm, including left sided heart tube as the two primitive heart tubes fuse (data not shown). *cPitx2* thus exhibits a left-right asymmetrical expression prior to the appearance of morphological asymmetry. During heart looping (stage 11), *cPitx2* expression was still restricted in the left sided lateral mesoderm and was also detected in the left vitelline vein as well as the left half of the epimyocardium (Fig. 2c and 3c). In addition, *cPitx2* transcripts also present in the endoderm. By stage 15, expression was restricted to the atrial epimyocardium of the primitive heart (Fig. 2f). At later stages of development *cPitx2* was detected in the periocular mesenchyme, nasal pit, the brachial arch, dorsal root ganglia, as well as at the junction between the limb bud and body wall (Fig. 2d and data not shown). In addition, in extraembryonic tissues, *cPitx2* expression was detected in the presumptive blood islands of the area opaca as early as stage 5 (data not shown), and was also detected in the angioblasts of blood islands (Fig. 2h) and endothelial cells of the blood vessels (Fig. 2g) at later stages of development.

### *SHH Protein Activates Ectopic Expression of cPitx2*

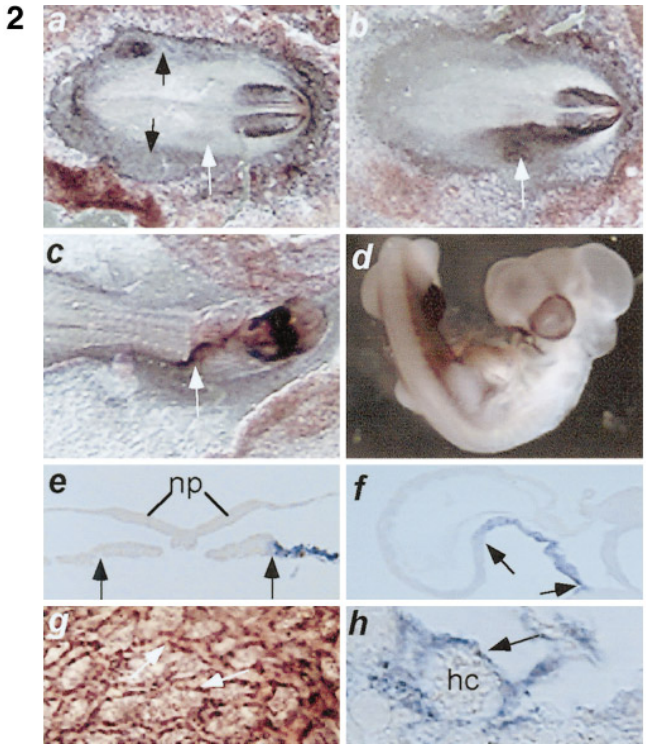
Since *cPitx2* expression is restricted to the left sided lateral mesoderm where *nodal* expression is induced by *Shh*, it is possible that *cPitx2* is also involved in the SHH signaling pathway which regulates heart looping. The effect of SHH on *cPitx2* expression in developing embryo was tested by in ovo and in vitro New culture techniques. To determine whether SHH can regulate *cPitx2* expression, beads soaked with SHH protein were implanted on either the left or right side of stage 4 or stage 5 chick embryos at the level of the node. Beads soaked with BSA were implanted to the right side of the same staged embryos as controls. Bead-implanted embryos were re-incubated and allowed to develop to stage 11 when they were collected, and *cPitx2* expression was examined by whole-mount in situ hybridization. These experiments demonstrated that ectopic SHH to the right side of the embryo can induce ectopic expression of *cPitx2* in the right sided lateral mesoderm, the right vitelline vein and the right side of the heart tube (Fig. 3b). Sectioning of these embryos revealed the expression of *cPitx2* in both the left and right sides of epimyocardium of the fused heart tube and in the lateral mesoderm on both sides of embryo (Fig. 3d,f). Control BSA beads implanted on the right side of embryos had no effect on *cPitx2* expression (Fig. 3a,c,e). In addition, SHH soaked beads implanted on the left side of embryos also did not affect *cPitx2* expression in the hosts (data not shown). These results indicate that *Shh* is upstream to *cPitx2* and may be responsible for setting up left-right asymmetric expression of *cPitx2* in the early chick embryogenesis.

## DISCUSSION

We report the isolation and characterization of an avian homeobox containing gene, *cPitx2*. *cPitx2* is 76% and 77% identical to its mouse and human homologues respectively. At the protein level, the homeodomain is 100% conserved between the mouse and the chick, and differs by one amino acid in the human RIEG homeodomain sequence. Upon examination of the expression pattern of *cPitx2* we found that at later stages of development (such as stage 21), expression of *cPitx2* mirrors the expression pattern previously reported in the mouse (16, 17), including the periocular mesenchyme, the first branchial arch and the junction between body wall and the limb buds. Thus, the highly conserved expression pattern of *cPitx2* in mice and chicken agrees with its sequence conservation.

The most striking expression pattern of *cPitx2* is its asymmetric expression along the left-right axis in the early developing embryo. This asymmetric expression has not yet been reported in mice. In chicken embryos, this asymmetrical expression of *cPitx2* starts as early as stage 7 and by stage 8 expression is restricted to left side lateral mesoderm including left side precardiac mesoderm. As development proceeds, the asymmetric expression of *cPitx2* is maintained and becomes evident in the left sided migrating heart primordia, the left vitelline vein and the left half epimyocardium of the fused heart tube. After dextral looping of the heart tube, *cPitx2* expression becomes restricted to the atrium of the primitive heart. These results strongly suggest that *cPitx2* is involved in the determination of left-right asymmetry of the embryo and in rightward heart looping. It would be very interesting to examine whether this asymmetric expression pattern of *Pitx2* is also conserved in mice.

Previous studies have already proposed a model for the determination of left-right asymmetry and dextral looping of the heart (10-12). This model involves a number of signaling molecules, in particular, the putative morphogens SHH and Nodal. *Shh*, which becomes asymmetrically expressed in the node by stage 5, is responsible for the asymmetric expression of *nodal* to the left side lateral plate mesoderm (10, 13). Additionally, missexpression of *nodal* to the right side of the chick embryo also results in randomized heart looping (11). Thus, the *Shh-nodal* pathway has been suggested to be a key player in the determination



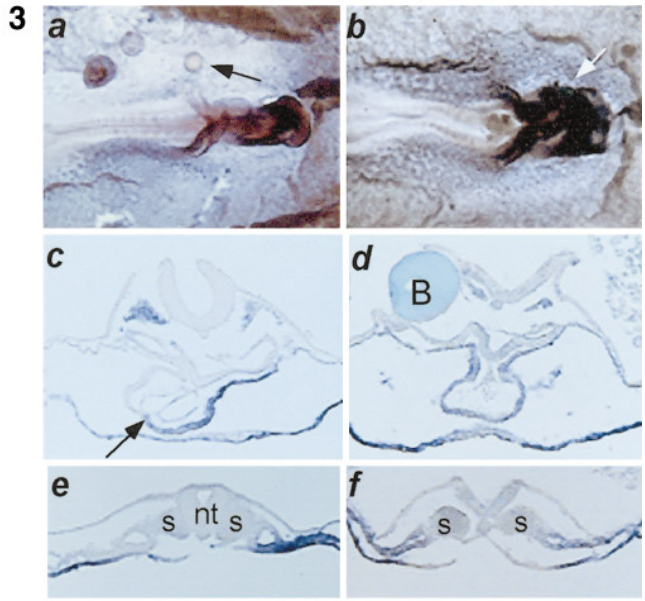




**FIG. 2.** Expression of *cPitx2* in developing chicken embryos. (a): Ventral view of a stage 7 chick embryo showing the first sign of asymmetric expression of *cPitx2* in the left sided lateral plate mesoderm, as indicated by the black arrow. Expression is also detected in area opaca vasculosa (white arrows) and in the head mesenchyme. (b): Ventral view of a stage 8 embryo showing the broadening domain of *cPitx2* expression on the left side (arrow). (c): Ventral view at stage 11 showing *cPitx2* expression in the left sided lateral mesoderm and the left vitelline vein (arrow). (d): Dorsal view of stage 21 embryo showing *cPitx2* expression in the periocular mesenchyme, nasal pit, the first brachial arch, dorsal root ganglia, and at the junction between the limb bud and body wall. (e): Anterior view of a section through a stage 8 embryo showing an asymmetrical expression in the left sided lateral mesoderm. (f): A section through a stage 15 embryo showing *cPitx2* expression in the presumptive atrium of the primitive heart. (g): Whole mount view of a stage 13 embryo indicating *cPitx2* expression in the endothelia of the blood vessels. (h): A section from a stage 10 embryo showing expression is restricted to the angioblasts of blood islands. np, neural plate; hc, hemopoietic cells.

**FIG. 3.** *cPitx2* expression is regulated by SHH. (a): Ventral view of a stage 11 embryo with a BSA bead (arrow) implanted at stage 4 to the right side of the embryo. No ectopic expression of *cPitx2* is observed in the embryo. (b): Ventral view of a stage 11 embryo with a SHH bead (arrow) implanted to the right side of the embryo at stage 4 showing ectopic expression of *cPitx2* to that side, including lateral mesoderm, vitelline vein and the right sided heart. (c): Anterior view of a section through heart region of a stage 11 control embryo indicating that *cPitx2* expression is restricted to the left side epimyocardium. Arrow points to the fusing boundary of left and right side heart tubes. Expression in the endoderm is also seen. (d): Anterior view of a section through heart region of a stage 11 embryo receiving a SHH bead on the right side showing the induced expression of *cPitx2* to the right side of the heart. (e): Anterior view of a section through a stage 11 control embryo showing asymmetrical expression of *cPitx2* to the left sided lateral mesoderm. (f): Anterior view of a section through a stage 11 embryo implanted with SHH bead on right side showing the induced symmetrical expression of *cPitx2* in both sides of the lateral plate mesoderm. B, bead; nt, neural tube; s, somite.

of left-right asymmetry and rightward heart looping. Our studies indicate that ectopic placement of SHH protein to the right side of the embryo induces ectopic expression of *cPitx2* to that side supports the idea that this gene is also a component in SHH signaling pathway. Since the asymmetrical expression of *cPitx2* appears slightly later than and overlaps with that of *nodal*, it is possible that *cPitx2* represents a downstream target of *Shh-nodal* pathway and executes asymmetrical morphology itself. Further studies, however, must be done to determine if *cPitx2* expression is directly regulated by SHH signal and/or is mediated by *nodal*. Our data also suggest that *cPitx2* may also be involved in vasculogenesis. This is evidenced by the fact that *cPitx2* expression is detected in the presumptive blood islands of the area opaca as well as the angioblasts of differentiated blood islands at the early stages of chick embryogenesis. As development proceeds, *cPitx2* transcripts localize to endothelial cells of blood vessels. These results indicate that *cPitx2* may play a role in the determination and differentiation of endothelial cells during embryogenesis. Further functional analysis is required to confirm this hypothesis.

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