Embryonic origins of spleen asymmetry

Kristin D. Patterson, Thomas A. Drysdale* and Paul A. Krieg†

Section of Molecular Cell and Developmental Sciences, University of Texas at Austin, Austin, TX 78712, USA

*Present address: Lawson Research Institute, St. Joseph’s Health Center, 268 Grosvenor Street, London, Ontario, N6A 4V2, Canada
†Author for correspondence at present address: Department of Cell Biology and Anatomy, University of Arizona College of Medicine, 1601 N. Campbell Avenue, Tucson, AZ 85724, USA (e-mail: pkrieg@u.arizona.edu)

Accepted 8 October; published on WWW 8 December 1999

SUMMARY

The spleen is a vertebrate organ that has both hematopoietic and immunologic function. The embryonic origins of the spleen are obscure, with most studies describing the earliest rudiment of the spleen as a condensation of mesodermal mesenchyme on the left side of the dorsal mesogastrium. The development of spleen handedness has not been described previously, presumably because of the difficulty in assaying spleen position in the embryo and the lack of early, organ-specific molecular markers. Here we show that expression of the homeobox gene \( \text{Nkx2-5} \) serves as a marker for spleen precursor tissue. Pre-splenic tissue is initially located in symmetric domains on both sides of the embryo but, during subsequent development, only the left side goes on to form the mature spleen. Therefore, the final location of the spleen on the left side of the body axis appears to result from preferential development of the spleen precursor cells on the left side of the embryo. Our studies indicate that the spleen and heart become asymmetric via different cellular mechanisms. \( \text{Nkx2-5} \) may function locally as part of the laterality cascade, downstream of \( \text{nodal} \) and \( \text{Pitx2} \), or it may direct asymmetric morphogenesis after laterality has been determined.

Key words: Left/right axis, Spleen, Nkx2-5, Homeobox gene, tinman, Polysplenia, Vertebrate

INTRODUCTION

Most visceral organs are asymmetric along the left/right axis. For example, in humans, the bulk of the liver is located on the right side of the body, and the left lung is bilobed, while the right lung is trilobed. Furthermore, in vertebrates, the heart and gut tubes undergo looping morphogenesis resulting in asymmetric mature organs. The spleen, a lymphoid organ that plays a role in hematopoiesis, the generation of some immune responses, and in the removal and processing of spent red blood cells (VanRooijen et al., 1989; Zapata and Cooper, 1990), is located on the left side of the vertebrate body. Very little is known about the early ontogeny of the spleen. Histological studies using frog, chick and mammalian embryos, however, indicate that the splenic rudiment is first recognizable as a single condensation of mesodermal mesenchyme along the left side of the mesogastrium dorsal to the stomach (Thiel and Downey, 1921; Nelsen, 1953; Manning and Horton, 1969; Sty and Conway, 1985; Vellguth et al., 1985; Yassine et al., 1989). Therefore, both the embryonic and mature spleen display handed asymmetry.

Although the pattern of organ asymmetries is very consistent within each species, deviations have been observed. In mice, the \( \text{situs inversus viscerum} \) mutation, \( \text{iv/iv} \), results in randomization of handedness (Layton, 1976). Approximately 50% of \( \text{iv/iv} \) embryos are normal and 50% are reversed along the left/right axis. However, heterotaxia, where some organs are reversed and others are not, is common in \( \text{iv/iv} \) mice (Seo et al., 1992). The gene mutated in \( \text{iv/iv} \) mice has been isolated and codes for an axonemal dynein (Supp et al., 1997). Another mutation, \( \text{inv/inv} \), results in complete reversal of organ handedness in 100% of the homozygous mice (Yokoyama et al., 1993). Laterality defects that result in left or right isomerism of visceral organs (Polysplenia and Asplenia Syndromes, respectively), as well as reversed axes have also been documented in humans (Burn, 1991).

The processes involved in the generation of left/right asymmetries have been divided into three phases: Orienting the left/right axis with respect to the anteroposterior and dorsoventral axes, asymmetric gene expression and asymmetric morphogenesis of specific organs (for review see Levin and Mercela, 1998). Significant progress has been made recently in identifying genes which are expressed asymmetrically along the left/right axis. In the chick embryo, sequences coding for a snail-related zinc-finger transcription factor (\( \text{cSnR} \)) (Isaac et al., 1997), a TGF\( \beta \) growth factor (\( \text{activin} \) \( \beta \beta \)) (Levin et al., 1997) and an activin receptor (\( \text{Act-RIIa} \)) (Levin et al., 1995) are expressed exclusively on the right side. Conversely, sequences coding for the signaling molecule sonic hedgehog (\( \text{shh} \)) (Levin et al., 1995), a winged-helix transcription factor (\( \text{HNF3-} \beta \)) (Levin et al., 1995), a nodal-related TGF\( \beta \) growth factor (\( \text{cNR1} \)) (Levin et al., 1995) and the homeodomain transcription factor (\( \text{Pitx2} \)) (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998)
are restricted to the left side. In addition, the TGFβ family members lefty1 and lefty2 are expressed on the left side of mouse embryos (Meno et al., 1996, 1997). Ectopic expression of several of these molecules, including shh, nodal, lefty, and Pitx2, influences the development of handedness (Levin et al., 1995, 1997; Logan et al., 1998; Pagan-Westphal and Tabin, 1998; Ryan et al., 1998; Yoshioka et al., 1998).

There is evidence that at least some of the laterality cascade is conserved in different vertebrates. cNR1 and its mouse and frog orthologues, nodal and XNR1 respectively, are expressed in the left lateral plate mesoderm during embryogenesis (Collignon et al., 1996; Lowe et al., 1996; Lohr et al., 1997). Expression of nodal on the right side of chick and frog embryos results in randomization (or isomerism) of heart and gut handedness (Sampath et al., 1997; Levin et al., 1997) and nodal expression is correlated with handedness in mouse mutants (Lowe et al., 1996). Pitx2 is normally expressed on the left side of the heart, visceral mesoderm and gut tube in mouse, chick and frog embryos. It is activated ectopically by nodal on the right side of chick and frog embryos, and misexpression of Pitx2 on the right side results in laterality reversals (Logan et al., 1998; Meno et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). Of the molecules isolated to date, Pitx2 is the most downstream in the laterality cascade. Although these studies suggest that at least some aspects of laterality are determined similarly in different vertebrates, the mechanisms required for specific visceral organs to interpret laterality signals have not been studied, presumably because of the difficulty in assaying the handedness of many organs in the embryo.

The study of spleen formation has been hindered by the absence of early molecular markers. Although the homeobox gene Hox11 is expressed in the condensing splenic mesenchyme and is required for spleen formation (Roberts et al., 1994; Dear et al., 1995), it is expressed rather late during development and does not serve as a marker for the study of early spleen development, prior to condensation. In this report, we show that the Xenopus tinman-related homeobox gene, XNks2-5, is expressed in spleen tissue in the adult frog and in the tadpole. It is initially expressed in symmetrical pre-splenic tissue and only later becomes asymmetric. This pattern of XNks2-5 expression permits a study of the generation of handedness in the spleen. The presence of symmetrical spleen precursors provides a potential explanation for the embryological mechanism underlying human polysplenia and asplenia syndromes.

MATERIALS AND METHODS

RNA analysis

Total RNA from adult organs was isolated by homogenization in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M β-mercaptoethanol, followed by addition of 1/10 volume 2 M sodium acetate pH 4.0, phenol/chloroform extraction and ethanol precipitation. The RNA was further purified by LiCl precipitation.

The template used to synthesize an antisense RNA protection probe specific to Xenopus XNks-2.5 (XNks-RVH) was constructed by inserting the 299 bp BglII-EcoRV fragment of the full-length XNks-2.5 cDNA (Tonissen et al., 1994) into plasmid T7TS. Labeled antisense probes were made by linearizing the template with HindIII and transcribing using SP6 RNA polymerase. EF-1α (Krieg et al., 1989) transcripts were quantified as controls. RNase protections were completed using the Ambion RPAII kit or the method described in Krieg (1991).

Embryo manipulation and in situ hybridization

Xenopus embryos were generated by injecting female frogs with 800 IU human chorionic gonadotrophin to induce egg laying, followed by fertilization in vitro. Embryos were staged according to Nieuwkoop and Faber (1994) and fixed in MEMPFA (4% paraformaldehyde, 100 mM MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO4) for 2 hours at room temperature or overnight at 4°C. Whole-mount in situ hybridization was performed as described in Harland (1991) with the following modifications: CHAPS was omitted from all buffers, Tris-buffered saline (50 mM Tris, pH 7.4, 200 mM NaCl) was used in place of phosphate-buffered saline, embryos were treated with 5 µg/ml proteinase K for 15 minutes, the 0.2× SSC washes were increased to 1 hour in length and final washes were increased to 12 at 30 minutes each. After the color reaction, embryos were rinsed 2× 5 minutes in alkaline phosphatase buffer, followed by dehydration through a methanol series and rehydration into water. The embryos were then fixed in Bouin’s fixative overnight at room temperature. Embryos were dehydrated into methanol for storage. For photography, embryos were cleared in 1:2 benzyl alcohol/benzyl benzoate. For sectioning, embryos were dehydrated in ethanol, permeabilized in xylene and embedded in Paraplast. Sections (10 µm) were dried, dewaxed in xylene, mounted with Permount and photographed using Nomarski optics.

Apoptosis assay

To assay for apoptosis, embryos between stages 37 and 40 were collected at 2 hour intervals. Six embryos at each stage were embedded in paraplast and sectioned. Dewaxed, unstained sections were rehydrated through an ethanol series, refixed for 20 minutes in MEMPFA, rinsed 3× in TBST, permeabilized in 25 µg/ml Proteinase K for 30 minutes at 37°C and rinsed 3× in TBST. Apoptosis was then detected using the TUNEL-protocol described in Conlon et al. (1995) except that the reaction mix contained 2 µM digoxigenin dUTP. Control embryos were used to approximate the location of the XNks2-5 expression region in the experimental embryos. XNks2-5 was detected by whole-mount in situ hybridization in several control embryos of each stage. These embryos were then sectioned identically to the experimental embryos. In the control embryos, the number of sections separating the heart from the XNks2-5 expression in the abdomen was determined. An equivalent region of the experimental embryos was examined for the presence of apoptotic cells.

Lineage tracing

For lineage tracing of embryos at stage 37, single crystals of diI were implanted on either the left or right sides, in the region expressing Nks2-5. At stage 40, cryostat sections (20 µM) of embryos were prepared and observed using epifluorescence to determine the location of diI-labeled cells. For lineage tracing using FDA, fertilized embryos were incubated in 4% Ficoll in Modified Danilchik’s Media without bovine serum albumin. When the first cleavage furrow was complete, one cell was injected with 4.6 nl of 100 mg/ml fluorescein-dextran-amine (FDA, Molecular Probes, D-1820) in distilled water. Embryos were cultured until stage 40, fixed in Bouin’s fixative overnight at room temperature or overnight at 4°C. Whole-mount in situ hybridization was performed as described in Harland (1991) with the following modifications: CHAPS was omitted from all buffers, Tris-buffered saline (50 mM Tris, pH 7.4, 200 mM NaCl) was used in place of phosphate-buffered saline, embryos were treated with 5 µg/ml proteinase K for 15 minutes, the 0.2× SSC washes were increased to 1 hour in length and final washes were increased to 12 at 30 minutes each. After the color reaction, embryos were rinsed 2× 5 minutes in alkaline phosphatase buffer, followed by dehydration through a methanol series and rehydration into water. The embryos were then fixed in Bouin’s fixative overnight at room temperature. Embryos were dehydrated into methanol for storage. For photography, embryos were cleared in 1:2 benzyl alcohol/benzyl benzoate. For sectioning, embryos were dehydrated in ethanol, permeabilized in xylene and embedded in Paraplast. Sections (10 µm) were dried, dewaxed in xylene, mounted with Permount and photographed using Nomarski optics.

Generation of axis reversals

To generate axis reversals, embryos were treated with UV light for 10, 15 or 20 seconds at the 1-cell stage. The resulting embryos were...
sorts according to the DAI scale (Kao and Elinson, 1988). The direction of heart looping in DAI 3–4 embryos was determined at stage 37, before fixation. Embryos with reversed and normal heart looping were fixed at stage 41 in MEMPFA. The direction of gut looping was determined by visual inspection and the position of XNkx2-5 expression was assayed by in situ hybridization. To generate axis reversals using octopamine, dechorionated embryos were incubated in 10% Steinberg’s medium containing 0.05 mM octopamine (Sigma) from stage 18 onwards as described by Toyozumi et al. (1997). The direction of heart and gut looping and the location of XNkx2-5 expression were determined as described for UV-treated embryos.

RESULTS

XNkx2-5 is expressed in symmetrical pre-splenic tissue

In mouse (Lints et al., 1993) and in Xenopus (Fig. 1A), the Nkx2-5 homeobox gene is expressed at high levels in the adult heart and spleen, but is barely detectable in other visceral tissues. In late stage Xenopus embryos, whole-mount in situ hybridization analysis reveals Nkx2-5 expression in the developing spleen, at a time when it is distinguishable as a condensation of mesenchyme on the dorsal side of the stomach (Nieuwkoop and Faber, 1994; Fig. 1B,C). In earlier stage Xenopus embryos, Nkx2-5 expression can be traced back to a crescent-shaped region of visceral mesoderm overlying the foregut on the left-hand side of the stage 37 embryo (Fig. 2A). However, at stage 37 an identical region of Nkx2-5-expressing cells is also present on the right-hand side (Fig. 2B). During subsequent development, Nkx2-5 expression on the right gradually diminishes, becoming undetectable by stage 40 (Fig. 2F,J). On the left side, the Nkx2-5-expressing cells move ventrally, becoming semi-tubular in shape as the foregut tube forms and begins to loop (Fig. 2E,J), finally residing in the location of the mature spleen. This pattern is highly consistent, with Nkx2-5 expression being maintained on the left side in 95% of embryos examined. The remaining 5% of embryos either lack Nkx2-5 entirely in this region or maintain Nkx2-5 on the right side. In the small number of embryos where Nkx2-5 transcripts are observed on the right side, the direction of foregut looping is also reversed. It is important to acknowledge that the observation of a moving domain of Nkx2-5 expression at different developmental stages does not necessarily mean that the cells are migrating. Although movement of cells expressing Nkx2-5 is the more economical hypothesis, it is strictly possible that different cells express Nkx2-5 at different developmental stages.

Spleen asymmetry is generated through a novel mechanism

These observations of Nkx2-5 expression in the frog embryonic viscera suggest that the embryo initially contains two symmetrical regions of pre-splenic tissue, but that only tissue on the left side ultimately contributes to the mature organ. There are a number of mechanisms by which Nkx2-5 expression could become asymmetric along the left-right axis. First, the precursors might migrate and fuse, as occurs during heart tube formation. Second, the precursor pool on the right side may undergo differential programmed cell death. Finally, the cells on the right might assume different developmental potentials due to subsequent embryonic signals.

In the frog embryo, Nkx2-5 transcripts disappear from the right side over a 10 hour period, between stages 37 and 40. Examination of embryos at 2 hour intervals during this time indicates that Nkx2-5-expressing cells on the left side move ventrally, while those on the right move dorsally, following the rotation of the foregut (Fig. 2C,G,K). Histological sections through stage 37 embryos shows that the two regions of Nkx2-5 expression are symmetrical and well separated (Fig. 2D). Later, the Nkx2-5 expression on the right side becomes more condensed as the expressing cells are trapped in a pocket created by the looping foregut (Fig. 2H). Finally, the staining on the right side disappears completely, while the Nkx2-5-expressing cells on the left side remain adjacent to the forming gut tube (Fig. 2L). Based on in situ hybridization experiments therefore, there is no evidence for migration of Nkx2-5-expressing cells from the right side to the left side of the embryo. To examine this question more closely, we have carried out two additional cell labeling studies. First, diI was used to label, as accurately as possible, the Nkx2-5-expressing regions on either the left or right sides of stage 37 embryos. At stage 41, after the loss of visible Nkx2-5 expression on the right side (Fig. 2L), the embryos were viewed using fluorescence. When cells on the left were marked, diI-labeled cells were observed to move along with the rotating foregut. When diI was applied on the right side of the embryo, movement of the labeled tissue once again corresponded with rotation of the gut tube, but no migration of cells from the right to the left was observed (20 embryos examined – data not shown). Second, to ensure that all cells on the right side of the embryo with potential to contribute to the spleen were marked, the right cell of a 2-cell embryo was injected with FDA. Any migration of cells from right to left should be visible as FDA-stained tissue on the left side at stage 40-41. In 50 embryos examined, it was common to observe scattered labeled epidermal cells on the left side, but no migration of other cells to the left side was detected (data not shown). Overall, these results strongly suggest that the Nkx2-5-expressing cells on the right side of the embryo do not migrate to fuse with the putative pre-splenic tissue on the left.

Do the XNkx2-5-expressing cells disappear from the right side due to apoptosis? To test this possibility, the presence of apoptotic cells was assayed in stage 37-40 embryos using TUNEL (Conlon et al., 1995), a procedure that has previously been used successfully for analysis of Xenopus embryos (Ishizuya-Oka et al., 1997; Yaoita and Nakajima, 1997; Ishizuya-Oka and Ueda, 1996; Nishikawa and Hayashi, 1995). Some apoptotic cells are detected near the eye (Fig. 3A) and in the neural tube (data not shown). The positively staining nuclei are small relative to control nuclei, suggesting that they are genuine apoptotic cells. The overall level of cell death observed in the abdomen is low (one to several cells per section) and we detect no increase in cell death on the right side of the abdomen relative to the left side between stage 37 and 40 (Fig. 3B; data not shown). It seems unlikely therefore, that the loss of Nkx2-5 staining from spleen precursors on the right side is due to programmed death of the expressing cells.

Nkx2-5 expression in explants

Since Nkx2-5 expression on the right side does not seem to be due to cell migration or apoptosis, it is likely that Nkx2-5-expressing cells on the right lose competence to become spleen
or the ability to respond to spleen induction signals. To determine whether Nkx2-5 expression is downregulated on the right side in response to an active signal, small regions of tissue containing Nkx2-5-expressing cells, as well as the underlying endoderm and overlying ectoderm, were explanted at stages 28, 33 and 37. It was not possible to accurately dissect Nkx2-5-expressing tissue from later stage embryos, due to the large-scale movement associated with gut morphogenesis. The explants were cultured until control embryos reached stage 41 and then assayed for Nkx2-5 expression (Fig. 4). In all of these experiments, explants from both sides continue to express Nkx2-5 until at least stage 41, long after the equivalent cells on the right, in vivo, have ceased expression. This suggests that tissue-interactions occurring after stage 37 are responsible for downregulation of Nkx2-5 expression on the right side of the embryo.

XNkx2-5 expression in embryos with axis reversals

Treatment of 1-cell stage Xenopus embryos with a mild dose of UV light results in a truncation of the dorsoventral axis and...
randomization of heart looping (Danos and Yost, 1995). We have exposed embryos to UV light and then assayed for gut, spleen and heart handedness. The results obtained in these experiments are presented in Table 1. The percentage of embryos with reversed heart looping (33% of combined DAI 3-4 embryos) is comparable to previous studies (Danos and Yost, 1995; Lohr et al., 1997). Our results indicate that other aspects of laterality are also influenced by treatment with UV light. In pre-splenic tissue, Nkx2-5 expression is maintained on the left side in 46% of embryos and maintained on the right in 37% of embryos. The remaining embryos exhibited isomerization, with Nkx2-5 maintained on both sides in 6% of the cases and on neither side in 11%. The direction of heart looping was random with respect to Nkx2-5 expression (Table 1).

Table 1. Location of spleen primordia (marked by Nkx2-5 expression) in embryos treated with UV light

<table>
<thead>
<tr>
<th>Direction</th>
<th>Total embryos</th>
<th>Location of Nkx2-5 expression (number of embryos)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>right</td>
</tr>
<tr>
<td>Heart looping</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>31</td>
<td>11</td>
</tr>
<tr>
<td>reversed</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Foregut looping</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>reversed</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

In embryos with normal chirality, both heart and foregut loop to the left. The sidedness of Nkx2-5 expression is randomized in UV-treated embryos, as are the directions of heart and foregut looping. The sidedness of Nkx2-5 expression is correlated with foregut looping, not heart looping. Statistical analysis using the Chi-squared test indicates that the correlation of Nkx2-5 expression with direction of gut looping is significant with a P value of 1x10^-12.

Fig. 3. Detection of apoptotic cells in histological sections. Transverse sections through a stage 39 embryo showing apoptotic cells at the level of the eye (A) and the foregut (B). In no case was a difference observed in the level of apoptotic cells between the left and right sides in the foregut between stages 37 and 40. a, apoptotic cells; cg, cement gland; e, eye; m, melanocytes.

Fig. 4. XNkx2-5 expression is maintained in explants of pre-splenic tissue. (A) Tissue explanted from the left side at stage 28. (B) Tissue explanted from the right side at stage 28. All explants were cultured until stage 41, then assayed by in situ hybridization using an XNkx2-5 probe. Note that explants from both sides of the embryo continue to express Nkx2-5.

Fig. 5. The handedness of foregut looping and XNkx2-5 expression are correlated in UV-treated embryos. (A) Side-view of a stage 40 individual with reversed foregut looping, reversed XNkx2-5 expression and normal heart looping. (B) Lateral view of a stage 40 individual with normal foregut looping, normal XNkx2-5 expression and reversed heart looping. (C) Stage 41 embryo with reversed XNkx2-5 expression and foregut looping. (D) Stage 41 embryo with normal XNkx2-5 expression and normal foregut looping. C and D were photographed at an angle between the ventral and lateral sides so that both the looping foregut and XNkx2-5 expression are visible. Arrows indicate XNkx2-5 expression in the spleen. XNkx2-5 is also expressed in the heart (h) in these embryos, although it is sometimes obscured by the liver (L).

Fig. 6. Bilateral XNkx2-5 expression in UV-treated embryos. In situ hybridization of a UV-treated embryo in which XNkx2-5 expression is maintained on both the right and left sides at stage 41. The embryo is viewed from the ventral side. h, heart; r, right; l, left.
(the normal side) is generally larger than that on the right (Fig. 6). These experiments show that, although the generation of asymmetry of the mesodermal spleen and the endodermal foregut appears to be coupled, the large-scale movements associated with gut looping are not required to generate asymmetric XNkx2-5 expression.

UV treatment is a rather crude method for generation of left/right axis reversals and interpretation of the results is complicated by the rather generalized disruption of normal embryonic development. For example, in the majority of UV-treated embryos, gut looping fails to occur in either direction. To more closely examine the relationship between spleen handedness (as marked by Nkx2-5 expression) and gut looping, we generated axis reversals by treating embryos with the adrenergic neurotransmitter, octopamine (Toyoiizumi et al., 1997). Octopamine effectively randomizes the direction of heart and gut looping without the more general developmental defects associated with UV treatment. Also, with the exception of left-right asymmetry reversals, the development of octopamine-treated embryos, including foregut looping, is apparently normal. The results obtained from octopamine treatment are summarized in Table 2. Of 132 treated embryos, 52 (39%) displayed reversed foregut situs and the sidedness of Nkx2-5 expression was also reversed in the great majority (96%) of these embryos. The converse is also true. Of the 80 embryos with normal foregut looping, 96% had Nkx2-5 transcripts restricted to the left side.

In five embryos, Nkx2-5 expression was apparently absent altogether, a percentage similar to that observed in control embryos. Foregut looping did occur in these embryos, three in the normal direction and two in the reversed direction. This experiment suggests that Nkx2-5 expression and foregut looping are strongly coupled.

When Nkx2-5 expression is observed on both sides in UV-treated embryos (Fig. 6), the appearance of the two groups of cells is consistent with that of pre-splenic mesenchymal cells, suggesting that Nkx2-5-expressing cells on the right-hand side of embryos with laterality defects are genuine spleen precursor cells with the potential to form a second spleen rudiment. Unfortunately, the embryos die before mature spleen structure is formed. To confirm the hypothesis that both groups of Nkx2-5-expressing tissue have the potential to form spleen, an independent marker of differentiated or functional spleen tissue, which temporally overlaps Nkx2-5 expression, is required. Previous studies have determined that the Xenopus spleen becomes immunologically competent at stage 50 (Manning and Horton, 1969) and, from this stage onwards, components of the immune system can be used as markers of spleen formation (Hadji-Azimi et al., 1982). Unfortunately, both explants of spleen tissue, and embryos with Nkx2-5 expression on both sides, begin to degenerate at about stage 45, before immune system markers are expressed. Alternatively, the homeobox gene Hox11 is an early marker of condensing spleen tissue during mouse development (Roberts et al., 1994; Dear et al., 1995). In Xenopus, the spleen begins to condense at stage 43 (Nieuwkoop and Faber, 1944) or later (Manning and Horton, 1969). We have isolated the Xenopus homologue of Hox11 and have determined its expression pattern during Xenopus embryogenesis (Patterson and Krieg, 1999). Unfortunately, Hox11 transcripts are not detectable in the Xenopus spleen before stage 45. Therefore, we have not been able to confirm that Nkx2-5-expressing cells on the right side of UV-treated embryos, or in explants, actually become differentiated spleen tissue.

**DISCUSSION**

**Nkx2-5 and the development of spleen asymmetry**

Using Nkx2-5 as a marker of tissue competent to become spleen, it appears that pre-splenic tissue is initially symmetrically located on each side of the embryo. A single left-sided spleen appears to be formed from precursor cells by a mechanism that involves neither migration of precursors nor differential cell death. Instead, the pre-splenic tissue on the right side of the embryo seems to adopt a different developmental program, leaving the precursors on the left to form the mature spleen. This process is strikingly different from the mechanism leading to the generation of asymmetry in other organs, including the heart and gut. Unlike the spleen, two symmetrical groups of precardiac cells migrate and fuse at the ventral midline to form the linear heart tube. The heart tube then undergoes looping morphogenesis to generate the characteristic handed asymmetry of the mature heart.

Randomization of the embryonic left/right axis by UV treatment indicates that the handedness of the heart and spleen are not linked in embryos with laterality defects. In contrast, the location of the spleen is strongly correlated with the direction of gut rotation and the location of the pancreas. This is similar to the situation in the iv/hv mouse, in which organ asymmetries are randomized but the handedness of the spleen and heart are not correlated (Seo et al., 1992). It is also consistent with human polysplenia syndrome, in which infants are generally bilaterally left-sided, but do not always suffer from cardiac isomerism (Debich et al., 1990). Although the spleen is derived from mesoderm, and the foregut and pancreas are endodermal, the three organs are intimately associated with each other spatially. The heart is nearby, but separated from the gut by the liver. Given the spatial relationships between the organs, the lack of a correlation in heart and spleen asymmetry suggests that the morphological handedness of specific organs may depend on local embryonic signals.

The generation of left/right asymmetry in the heart and spleen occurs at different times. In Xenopus, the generation of handedness is dependent upon dorsal axial structures (Danos and Yost, 1996). Removal of dorsal axial tissue from early

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**Table 2. Location of spleen primordia (marked by Nkx2-5 expression) is correlated with the direction of foregut looping in octopamine-treated embryos**

<table>
<thead>
<tr>
<th>Direction</th>
<th>Total embryos</th>
<th>Location of Nkx2-5 expression (number of embryos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal heart/normal gut</td>
<td>76</td>
<td>right 73 left 0 both 0 neither 3</td>
</tr>
<tr>
<td>reversed heart/normal gut</td>
<td>4</td>
<td>0 4 0 0</td>
</tr>
<tr>
<td>normal heart/reversed gut</td>
<td>20</td>
<td>0 20 0 0</td>
</tr>
<tr>
<td>reversed heart/reversed gut</td>
<td>32</td>
<td>30 0 0 0</td>
</tr>
</tbody>
</table>

In embryos with normal chirality, both heart and foregut loop to the left. Statistical analysis using the Chi-squared test indicates that the correlation of Nkx2-5 expression with direction of gut looping is significant with a $P$ value of $1 \times 10^{-29}$. 
neural tube embryos results in randomization of heart looping, whereas heart development proceeds normally when this experiment is completed after neural tube stages (Danos and Yost, 1996). Therefore, the direction of heart looping is dependent upon dorsal axial tissue until neural tube stages (stage 19). Similar results are achieved when heart tissue is explanted from the embryo, cultured and subsequently assayed for handedness (Danos and Yost, 1996). In contrast, when left and right spleen progenitors are explanted from the embryo as late as stage 37, Nkx2-5 transcripts are maintained on both sides. This experiment indicates that signals from other regions of the embryo are required to downregulate Nkx2-5 on the right side. Furthermore, these signals are not active until at least stage 37, much later than the signals responsible for asymmetric heart development.

There are at least three explanations for the correlation of spleen asymmetry (marked by Nkx2-5 expression) with the direction and timing of gut looping. First, the establishment of spleen asymmetry may be dependent on rotation of the gut, rather than on independent interpretation of laterality signals. If this is the case, embryos in which the gut fails to loop should display bilateral Nkx2-5 expression due to a failure to downregulate Nkx2-5 on the right side. Our results indicate that this is not the case in UV-treated embryos, where all possible combinations of Nkx2-5 expression were seen in embryos with linear gut tubes (Table 1). This observation indicates that the generation of unilateral Nkx2-5 expression depends on the laterality cascade, although we do not know which other asymmetrically expressed molecules may regulate Nkx2-5. Second, it is possible that the direction of gut looping is dependent on asymmetric Nkx2-5 expression. In support of this hypothesis, mice lacking Nkx2-5 gene products fail to undergo heart looping, resulting in a linear heart tube (Lyons et al., 1995). Perhaps, Nkx2-5 is also required to generate asymmetries in the foregut. We have observed a small number of embryos that lack Nkx2-5 expression yet undergo gut looping in the normal or reversed direction. These embryos may genuinely lack Nkx2-5 expression, suggesting that Nkx2-5 function is not required for the process of gut morphogenesis, or Nkx2-5 may be expressed asymmetrically in the foregut region at levels undetectable by in situ hybridization. Finally, asymmetrical Nkx2-5 expression and foregut looping may be generated independently in response to a common local signal.

**Relationship between Nkx2-5 and the laterality cascade**

Previous studies have characterized the expression of other members of the laterality pathway in normal embryos and in embryos with laterality defects (for reviews see King and Brown, 1997; Levin, 1997; Harvey, 1998; Levin and Mercora, 1998; Varlet and Robertson, 1997). In normal mouse, chick and Xenopus embryos, nodal transcripts are expressed asymmetrically in the left lateral plate mesoderm adjacent to the cardiogenic plate (Levin et al., 1995; Collingnon et al., 1996; Lowe et al., 1996; Lohr et al., 1997). In addition, altered nodal expression is correlated with defects in heart looping. For example, in chick and frog embryos, bilateral nodal expression results in randomization of heart asymmetry, including a high frequency of left-isomerism (Sampath et al., 1997; Levin et al., 1997). In UV-treated Xenopus embryos, XNR1 is expressed in the lateral plate mesoderm on both sides of the embryo and the direction of heart looping is randomized (Lohr et al., 1997). Mouse laterality mutants also show a different pattern of nodal expression. In inv/inv mutants, for example, transcripts are detected exclusively on the right side (Collingnon et al., 1996; Lowe et al., 1996). In iv/iv mutants, nodal expression is isomerized (either expressed bilaterally or not expressed on either side) in 60% of homozygous mice, but is sometimes detected only on the left or right side (Lowe et al., 1996). These results have led to the conclusion that nodal provides left-specific information in the embryo. Expression of the homeobox gene Pitx2 is downstream of nodal in the left lateral plate mesoderm in mouse, chick and frog embryos (Logan et al., 1998; Meno et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). Like nodal, Pitx2 is expressed in all combinations in embryos with laterality defects, with bilateral expression being the most prevalent (Piedra et al., 1998; Ryan et al., 1998). Most importantly, changes in Pitx2 expression correlate with alterations in organ handedness. Unlike the early downregulation of nodal, Pitx2 expression is maintained throughout organogenesis, suggesting that this molecule may provide laterality signals to developing organs.

Does Nkx2-5 function as part of the laterality cascade? Although Nkx2-5 transcripts are detected in the visceral mesoderm after the onset of Pitx2 expression (Fig. 2; Ryan et al., 1998), the acquisition of asymmetric Nkx2-5 is unlikely to be generated directly by Pitx2 for several reasons. First, Pitx2 transcripts are restricted to the left lateral plate mesoderm and Nkx2-5 is initially activated on both sides of the embryo. Secondly, the expression of Nkx2-5 in embryos with randomized axes does not correlate with Pitx2 (or nodal) expression (Lohr et al., 1997; Piedra et al., 1998; Ryan et al., 1998). Pitx2 and nodal transcripts are isomerized in a high percentage of randomized embryos, while Nkx2-5 expression is rarely isomerized and is usually expressed on either the left or right side. In addition, most organs develop asymmetrically in randomized embryos. Therefore, despite the bilateral expression of Pitx2 and nodal in embryos with laterality defects, Nkx2-5 is expressed asymmetrically and organs undergo asymmetric morphogenesis. Therefore, it is possible that Nkx2-5 functions as a local signal, downstream of nodal and Pitx2 in the laterality pathway, in such a way that Nkx2-5 expression is tightly linked with the handedness of the spleen and foregut. Alternatively, it may be involved in the morphogenesis of these organs after laterality has been determined.

**Symmetrical spleen precursors and human laterality defects**

The presence of symmetrical spleen precursors has not been reported previously, however, the existence of potential spleen precursors on the right-hand side of the embryo may help to explain defects observed in human spleen development. In human infants, bilateral right sidedness is associated with asplenia (lack of a spleen) and bilateral left sidedness is associated with polysplenia (multiple or multilobed spleens) (Burn, 1991). The presence of multiple spleens has often been attributed to abnormal development of a single splenic rudiment; i.e. the failure of initially separate condensates to fuse, or exaggeration of fissures that form during the normal course of spleen development (Moller et al., 1967; Arey, 1974). The observation that symmetrical cell groups are initially...


