Zic3 is involved in the left-right specification of the Xenopus embryo

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SUMMARY

Establishment of left-right (L-R) asymmetry is fundamental to vertebrate development. Several genes involved in L-R asymmetry have been described. In the Xenopus embryo, Vg1/activin signals are implicated upstream of asymmetric nodal related 1 (Xnr1) and Pitx2 expression in L-R patterning. We report here that Zic3 carries the left-sided signal from the initial activin-like signal to determinative factors such as Pitx2. Overexpression of Zic3 on the right side of the embryo altered the orientation of heart and gut looping, concomitant with disturbed laterality of expression of Xnr1 and Pitx2, both of which are normally expressed in the left lateral plate mesoderm. The results indicate that Zic3 participates in the left-sided signaling upstream of Xnr1 and Pitx2. At early gastrula, Zic3 was expressed not only in presumptive neuroectoderm but also in mesoderm. Correspondingly, overexpression of Zic3 was effective in the L-R specification at the early gastrula stage, as revealed by a hormone-inducible Zic3 construct. The Zic3 expression in the mesoderm is induced by activin β or Vg1, which are also involved in the left-sided signal in L-R specification. These findings suggest that an activin-like signal is a potent upstream activator of Zic3 that establishes the L-R axis. Furthermore, overexpression of the zinc-finger domain of Zic3 on the right side is sufficient to disturb the L-R axis, while overexpression of the N-terminal domain on the left side affects the laterality. These results suggest that Zic3 has at least two functionally important domains that play different roles and provide a molecular basis for human heterotaxy, which is an L-R pattern anomaly caused by a mutation in human ZIC3.

Key words: Left-right axis determination, Left-right asymmetry, Heterotaxy, Zic3, Vg1, activin, Pitx2, Xnr1, Xenopus

INTRODUCTION

Establishment of the left-right (L-R) asymmetry of the vertebrate internal organ is one of the most fascinating topics in developmental biology (Harvey, 1998; Ramsdell and Yost, 1998; Capdevila et al., 2000). Several genes involved in the establishment of the L-R axis have been characterized in various species. In the Xenopus embryo, various L-R asymmetry regulatory models have been proposed, and there appears to be two stages in this process, which is based on the sequential expression of known genes.

In the initial stage, members of the TGFβ superfamily are considered to act as L-R coordinators in the Xenopus embryo (Hyatt et al., 1996; Hyatt and Yost, 1998; Ramsdell and Yost, 1999). Misexpression of Vg1 or activin in the right blastomere can disturb the L-R patterning of the visceral organs and heart, whereas Bmp2 and Bmp4 influence the L-R axis only if misexpressed in the left side, suggesting that Vg1/activin and Bmp2/Bmp4 are involved in the left- and right-sided signaling pathways, respectively. Some reports have suggested that the gap junction is also involved in the L-R axis establishment in early Xenopus development (Levin and Mercola, 1998, 1999). Laterality defects have been induced either by blocking gap junction communication dorsally or by introducing gap junction communication ventrally. Since L-R asymmetric expression of TGFβ family genes is not found in the Xenopus embryo, the mechanism of induction of the later asymmetric genes by the early symmetric genes is still unclear.

The late stage of the L-R asymmetry pathway is conserved among many species. The roles of nodal related 1 (Xnr1) and Pitx2 have been investigated extensively (Sampath et al., 1997; Ryan et al., 1998; Campione et al., 1999). In the Xenopus embryo, both genes are expressed in the left lateral plate mesoderm (LPM), which is disturbed by misexpression of Vg1/activin on the right side of the embryo. Ectopic expression of either gene on the right side disturbs the L-R orientation of multiple organs. Xnr1 is considered to regulate Pitx2, since the asymmetric expression of Xnr1 precedes that of Pitx2, and the ectopic expression of Xnr1 induces Pitx2 expression at a corresponding site. The expression of Pitx2 continues throughout the process of heart development or gut looping and is considered to be a determinant of organ asymmetry.

In addition to Xnr1 and Pitx2, an activin-like signal also plays a role in the late stage of Xenopus embryo development...
A hormone-inducible construct of Zic3 (Zic3-GR), was also injected with lacZ mRNA into the dorsal right blastomere of the four-cell stage embryo. Injected embryos were first cultured without dexamethasone. At various stages, the medium was replaced by fresh medium containing dexamethasone, in which the embryos were kept until stage 25. Misexpression of Pitx2 was scored for these embryos after determination of the injected side by β-gal staining. The medium consisted of dexamethasone, which was added at a final concentration of 1 µM in 0.1× Steinberg’s solution.

To observe the effects of Xenopus Zic3 or Xenopus activin β on heart and gut looping, mRNA was injected into the left or right ventral blastomere of four-cell stage embryos. The embryos were cultured until stage 47, and scored for heart and gut orientation.

For animal cap assay, mRNA was injected into the animal side of two blastomeres of two-cell stage embryos. Embryos were grown until stage 9 when the animal cap region was excised. The explants were cultured in 0.5× MMR until the sibling embryo reached stage 25.

Construction of deletion mutants and inducible version of Zic3
Deletion mutants of Xenopus Zic3 named XZ3d4 (amino acids 214-441), XZ3d6 (amino acids 1-214), and XZ3d7 (amino acids 214-385) were constructed by PCR amplification of the corresponding cDNA region (T. N., T. K., J. A. and K. M., unpublished). Zic3-GR was generated by fusing the coding region of Zic3 (amino acids 1-441) to a fragment encoding the hormone-binding domain of the human glucocorticoid receptor (amino acids 512-777) (Hollenberg et al., 1985) derived from p64T-Xbra-GR (Tada et al., 1997). These deletion and inducible constructs were cloned into pCS2+ vector (Turner and Weintraub, 1994) for mRNA synthesis. Sequencing analysis was performed to confirm the constructs.

RNA isolation and RT-PCR assay
Preparation of total RNA and RT-PCR assay were carried out as previously described (Suzuki et al., 1994). EF1α was used to monitor RNA recovery. The sibling control embryo served as a positive control. PCR was also performed with RNA that had not been reverse-transcribed to check for the DNA contamination. Some primer sequences were obtained from The Xenopus Molecular Marker Resource (http://vize222.ro.uteexas.edu/).

In addition, we designed the following primers for use in this study. Xnr1: 5′-AGTCAAGTCTCTTGGCAACC-3′, 5′-TCAATAACAACCTCTCATCT-CCC-3′, Pitx2: 5′-CTTCACGCTCCTTCCACT-3′, 5′-TCACACGGGTCTGTTC-3′.

RESULTS
Right-sided Zic3 overexpression alters heart and gut looping
To assess the involvement of Zic3 in L-R axis establishment, we first examined the effect of unilateral Zic3 overexpression on heart and gut laterality. Zic3 mRNA was injected into the left or right side of embryos. The embryos were cultured until stage 47 when the L-R laterality of the heart and gut was clearly observed. In the normal Xenopus tadpoles, the heart ventricle is situated on the left side with the outflow tract looping to the right side (Fig. 1A), and the gut coils counterclockwise (Fig. 1B). Following injections of Zic3 mRNA into the right side of the embryo, a significant number of the embryos showed situs abnormalities in the heart (Fig. 1C) and gut (Fig. 1D). The frequency was comparable with that caused by the right-sided injection of activin mRNA (Fig. 1E, Table 1). In contrast, injection of Zic3 mRNA into the left
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Zic3 regulates the asymmetric expression of Xnr1 and Pitx2

To determine the molecular basis of the above result, we next examined the expression of Xnr1 and Pitx2 with Zic3 overexpression in tailbud stage embryo. In most control embryos, Xnr1 and Pitx2 were expressed in the left LPM (Fig. 2A,B,E,F). When Zic3 was overexpressed on the right side, ectopic expression of both markers was observed in the right LPM (Fig. 2C,D,G,H). Overexpression of Zic3 on the left side caused bilateral or right LPM expression in fewer embryos (Fig. 2I; Table 2). Thus, the change in laterality of Xnr1/Pitx2 expression was correlated well with that of the heart and gut. Moreover, these results showed that Zic3 acts upstream of Xnr1 and Pitx2 in the establishment of the L-R axis.

The right-sided overexpression of Zic1 and Zic2 also disturbed the expression of Xnr1 and Pitx2 (Fig. 2IJ; Table 2), but to a lesser extent to that seen with Zic3 overexpression. Overexpression of another zinc-finger protein, GLI1, did not disturb the laterality of Xnr1 and Pitx2 expression, indicating that the ability to control L-R axis specification is specific to Zic family proteins.

Zic3 is symmetrically expressed in the mesoderm

We next examined Zic3 expression. However, we did not find L-R asymmetric expression of Zic3 at any stage (Fig. 3B,F,H; data not shown). We have previously shown that Zic3 is expressed in prospective neuroectoderm and dorsal lip at the early gastrula stage (Nakata et al., 1997). In addition to the

Table 1. Zic3 injection induces reversed heart and gut laterality

<table>
<thead>
<tr>
<th>Zic3 injected</th>
<th>activin</th>
<th>Normal</th>
<th>Reversed heart</th>
<th>Reversed gut</th>
<th>Reversed heart and gut</th>
<th>% Reversed organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>R</td>
<td>L</td>
<td>R</td>
<td>L</td>
<td>R</td>
<td>L</td>
</tr>
<tr>
<td>Normal</td>
<td>124 (16/124)</td>
<td>92 (18/92)</td>
<td>73 (9/73)</td>
<td>84 (3/84)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reversed heart</td>
<td>0</td>
<td>13 (7/13)</td>
<td>13 (6/13)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reversed gut</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reversed heart and gut</td>
<td>1</td>
<td>5</td>
<td>8</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Reversed organs</td>
<td>3</td>
<td>18</td>
<td>24</td>
<td>0</td>
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</tr>
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</table>

The number of abnormal morphology of gut looping are given in parentheses.

blastomere resulted in fewer in situ abnormalities, suggesting that Zic3 acts in the left signaling pathway of the L-R axis establishment process.

Table 2. Zic3 injection disturbs the expression of Xnr1 and Pitx2

(A) Xnr1 expression

<table>
<thead>
<tr>
<th>Zic3 injection</th>
<th>activin injection</th>
<th>Zic1 injection</th>
<th>Zic2 injection</th>
<th>GLI1 injection</th>
</tr>
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<tbody>
<tr>
<td>L</td>
<td>R</td>
<td>L</td>
<td>R</td>
<td>L</td>
</tr>
<tr>
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<td>59</td>
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<td>14</td>
<td>15</td>
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<td>Right</td>
<td>2</td>
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<td>2</td>
</tr>
<tr>
<td>Bilateral</td>
<td>19</td>
<td>25</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>% Altered expression</td>
<td>26</td>
<td>62</td>
<td>26</td>
<td>46</td>
</tr>
</tbody>
</table>

(B) Pitx2 expression

<table>
<thead>
<tr>
<th>Zic3 injection</th>
<th>activin injection</th>
<th>Zic1 injection</th>
<th>Zic2 injection</th>
<th>GLI1 injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>R</td>
<td>L</td>
<td>R</td>
<td>L</td>
</tr>
<tr>
<td>Left</td>
<td>65</td>
<td>32</td>
<td>24</td>
<td>23</td>
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<tr>
<td>Right</td>
<td>1</td>
<td>14</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Bilateral</td>
<td>2</td>
<td>14</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>% Altered expression</td>
<td>4</td>
<td>36</td>
<td>0</td>
<td>34</td>
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</table>
neuroectoderm, Zic3 expression was observed in the ring of involuting mesoderm (Fig. 3B,D) as indicated by the comparison with brachyury expression (Fig. 3A,C). Zic3 expression in mesoderm is stronger on the dorsal side. The mesodermal expression of Zic3 was diminished in the region surrounding the blastopore (Fig. 3F,H) where brachyury is expressed (Fig. 3E,G) at stage 12. Significant expression remained in the lateral mesoderm at stage 12 and was hardly detectable after stage 14 (data not shown). The expression of Zic3 in mesoderm, especially in the organizer region, may be related to the L-R specification, because the organizer plays a crucial role in the establishing the L-R axis (Danos and Yost, 1995). The expression of Zic1 and Zic2 is also observed in the dorsal mesoderm (Fig. 3K,L) in a L-R symmetrical fashion (data not shown). Taken together with the results of the overexpression experiments, the Zic family may be involved in L-R specification in a similar manner.

Zic3 was used in the following experiments as a representative Zic family gene because Zic3 showed the strongest L-R disturbing activity in the unilateral overexpression assay. Moreover, Zic3 is the only member that has been shown to be involved in the L-R specification process in other species.

**Overexpression of the zinc-finger domain or N-terminal domain of Zic3 is sufficient to disturb the L-R axis**

As a first step towards elucidating the molecular mechanism of Zic3-mediated L-R axis establishment, we performed a structure-function analysis using three deletion constructs. mRNA for XZ3d4 (zinc-finger domain and C-terminal domain), XZ3d6 (N-terminal domain) or XZ3d7 (zinc-finger domain) was injected into right or left blastomeres (Fig. 4A). The laterality was then assessed by the expression of Xnr1 and Pitx2 in the LPM. Overexpression of each of these constructs resulted in L-R disturbance (Fig. 4B,C; Table 3). Either the zinc-finger domain alone or the N-terminal domain alone was sufficient to alter the L-R axis, suggesting that there are at least two functionally

| Table 3. Deletion mutants of Zic3 can disturb the expression of Xnr1 and Pitx2 |

<table>
<thead>
<tr>
<th>(A) Xnr1 expression</th>
<th>Zic3 injection</th>
<th>XZ3d4 injection</th>
<th>XZ3d6 injection</th>
<th>XZ3d7 injection</th>
<th>Zic3+XZ3d6 injection</th>
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<td>R</td>
<td>L</td>
<td>R</td>
<td>L</td>
</tr>
<tr>
<td>Left</td>
<td>59</td>
<td>23</td>
<td>57</td>
<td>49</td>
<td>20</td>
</tr>
<tr>
<td>Right</td>
<td>2</td>
<td>12</td>
<td>0</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Bilateral</td>
<td>19</td>
<td>25</td>
<td>8</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>% altered expression</td>
<td>26</td>
<td>62</td>
<td>14</td>
<td>40</td>
<td>57</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B) Pitx2 expression</th>
<th>Zic3 injection</th>
<th>XZ3d4 injection</th>
<th>XZ3d6 injection</th>
<th>XZ3d7 injection</th>
<th>Zic3+XZ3d6 injection</th>
</tr>
</thead>
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<tr>
<td></td>
<td>L</td>
<td>R</td>
<td>L</td>
<td>R</td>
<td>L</td>
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<tr>
<td>Left</td>
<td>65</td>
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<td>25</td>
<td>27</td>
<td>38</td>
</tr>
<tr>
<td>Right</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>4</td>
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<tr>
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<td>9</td>
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</tr>
<tr>
<td>% Altered expression</td>
<td>4</td>
<td>36</td>
<td>7</td>
<td>33</td>
<td>0</td>
</tr>
</tbody>
</table>
Xenopus Zic3 and left-right asymmetry

important domains. Interestingly, XZ3d6 injection into the left blastomere disturbed the Xnr1 expression more severely than that into the right, while XZ3d4 or XZ3d7 more frequently disturbed the L-R axis when injected into the right side, similar to full-length Zic3. However, the effect of XZ3d6 was limited to the laterality of Xnr1, not Pitx2, expression. Although the frequency of disturbed expression of Xnr1 by XZ3d6 is comparable with that of full-length Zic3, the intensity of ectopic expression by XZ3d6 is weaker than that of full-length Zic3 (data not shown). We therefore hypothesize that XZ3d6 partially disturbs the L-R signaling pathway.

To clarify whether XZ3d6 has a dominant-negative effect on Zic3, XZ3d6 was co-injected with Zic3 (Fig. 4B,C, Table 3). Right side injection of XZ3d6 attenuated the laterality disturbance caused by Zic3 overexpression in the right side. Furthermore, the disturbance by left side XZ3d6 injection into the left side was rescued by co-injection of Zic3. The results indicate that XZ3d6 is a dominant-negative repressor of Zic3, and that the laterality disturbance by the left-sided XZ3d6 injection may result from a block of endogenous Zic3 activity.

**Zic3 is involved in the establishment of L-R asymmetry at early gastrula stage**

To examine when Zic3 is involved in regulation of L-R axis formation, we constructed the Zic3-GR construct, in which the hormone-binding domain of the human glucocorticoid receptor is fused to Zic3 protein (Fig. 5A). Glucocorticoid receptor fusion proteins have been used in the analysis of several proteins, including a Zic family protein (Hollenberg et al., 1993; Kolm and Sive, 1995; Tada et al., 1997; Kuo et al., 1998). Addition of dexamethasone caused the formation of pigment cell clusters in Zic3-GR-expressing embryo and the induction of neural marker in animal cap explants (data not shown). Since these phenotypes were typically found in Zic3 mRNA-injected embryos when used to perturb Zic3 expression at the early gastrula stage, we concluded that Zic3 is involved in the establishment of L-R asymmetry at this stage.

\[\text{Zic3 is expressed in the mesodermal tissues at early gastrula.}
\]
\[\text{Zic3 is expressed in the ring of involuting mesoderm (B,D) like the}
\]
\[\text{pan-mesodermal marker, brachyury (A,C) at stage 10.5. Arrows}
\]
\[\text{indicate blastopore. In C,D, embryos were cut along the broken line}
\]
\[\text{shown in (I). At stage 12, Zic3 is not detected in involuting}
\]
\[\text{mesoderm (F,H) in contrast to brachyury (E,G). White arrows}
\]
\[\text{indicate yolk plug. (G,H) Embryos were cut along the broken line in}
\]
\[\text{(J). Zic1 (K) and Zic2 (L) are also expressed in the mesodermal}
\]
\[\text{tissues at early gastrula. Arrows indicate blastopore. bp, blastopore;}
\]
\[\text{D, dorsal side; V, ventral side; yp, yolk plug.}
\]

**Fig. 3.** Zic3 is expressed in the mesodermal tissues at early gastrula.

**Fig. 4.** Zinc-finger domain or N-terminal domain of Zic3 alone can disturb the L-R axis. (A) Deletion constructs of Zic3 used in this experiment. Xnr1 or Pitx2 expression in Zic3- (100 pg), XZ3d4- (500 pg), XZ3d6- (1000 pg) or XZ3d7- (500 pg) injected embryos. The frequency of the disturbed Xnr1 (B) or Pitx2 (C) expression by left or right side injections.
Zic3 specifies the L-R laterality at early gastrula stage.
(A) Hormone-inducible construct of Zic3 used in this experiment. (B) Disturbed expression of Pitx2 in Zic3-GR (100 pg) injected embryo when dexamethasone was added at several stages. The frequency of the disturbed Pitx2 expression by the right-sided injections. DEX, dexamethasone; hGR, human glucocorticoid receptor.

Fig. 5. Zic3 specifies the L-R laterality at early gastrula stage. (A) Hormone-inducible construct of Zic3 used in this experiment. (B) Disturbed expression of Pitx2 in Zic3-GR (100 pg) injected embryo when dexamethasone was added at several stages. The frequency of the disturbed Pitx2 expression by the right-sided injections. DEX, dexamethasone; hGR, human glucocorticoid receptor.

Fig. 6. Vg1 and activin can induce Zic3 expression in the mesoderm. We next examined the relationship between Vg1/activin and Zic3. Both Vg1 and activin are candidates for the initial coordinator of the left signaling pathway in Xenopus embryos. When activin or Vg1 was overexpressed unilaterally, mesodermal expression of Zic3 was enhanced in the injected side (Fig. 6A). Consistent results were obtained by animal cap explant assay. When Vg1 or activin was overexpressed in the explant, Zic3 was induced as monitored by RT-PCR assay (Fig. 6B,C). Expression of the genes for the neural markers, neural cell-adhesion molecule and neurogenin, was not induced, but that of the mesodermal marker muscle actin was induced, indicating that the induction of Zic3 represents expression in mesodermal tissue not neuroectoderm. In addition to Zic3, Xnr1 and Pitx2 were also induced by Vg1/activin injection. Therefore, the left signaling cascade in the embryo may operate in animal cap explants.

DISCUSSION
Zic3 acts in the left signaling pathway
In the series of Zic3 overexpression assays, overexpression on the right side always caused a more frequent L-R pattern disruption than injection in the left side. It has been reported that overexpression of Xnr1 and Pitx2 on the right side of the embryo results in the L-R axis disturbance (Sampath et al., 1997; Ryan et al., 1998; Campione et al., 1999), and that activin/Vg1 mRNA injection into the right side of the embryo resulted in disturbance of the L-R axis (Hyatt et al., 1996; Hyatt and Yost, 1998; Ramsdell and Yost, 1999), suggesting that these genes and Zic3 share a similar role in L-R body axis development in Xenopus embryos.

In mice, mutations in Pitx2, Nodal, Smad2 or activin receptor IIB cause right pulmonary isomerism (Lu et al., 1999; Lin et al., 1999; Kitamura et al., 1999; Nomura and Li, 1998; Oh and Li, 1997). In chick, Pitx2, NRI, ActRIIa and activin B are involved in L-R asymmetry (Ryan et al., 1998; Logan et al., 1998; Piedra et al., 1998; Yoshioka et al., 1998; Levin et al., 1995, 1997). Therefore, the mechanism of L-R body axis establishment is well conserved, meaning that our results with the Xenopus unilateral overexpression assay are likely to be generally relevant. Based on analogy to these genes, we hypothesize that Zic3 is also involved in the signaling pathway that confers the left identity in various species, including humans, in which a ZIC3 mutation is responsible for heterotaxy (Gebbia et al., 1997).

Location of Zic3 in the L-R signaling cascade
We examined whether Vg1 and activin, which are potential L-R coordinators at the early stages of L-R specification, can...
induce Zic3 expression in mesodermal tissue. These factors induced the expression of Zic3 in both embryos and the explant tissue. Thus, it is possible that Vgl/activin acts as an upstream factor of Zic3 for L-R axis establishment.

Of the various downstream genes we tested, Xnr1 and Pitx2 showed altered expression in the LPM with Zic3 unilateral overexpression. Pitx2 is involved in organ asymmetry in several species. Xnr1 has also been shown to regulate the laterality of Pitx2 expression and its expression in LPM precedes that of Pitx2 (Campione et al., 1999). In our studies, Zic3 and Zic3 deletion mutants always caused a more frequent L-R disturbance in Xnr1 expression than in Pitx2 expression. This is particular to XZ3d6, which disturbs Xnr1 expression, but has no influence on the Pitx2 expression. These observations may reflect a more determinative action of Pitx2 in the establishment of the L-R axis.

The Zic3-GR construct revealed that Zic3 is involved in the L-R asymmetry at early gastrula stage. The expression of Vgl and activin β is maternal (Melton, 1987; Suzuki et al., 1994), and the asymmetric expression of Xnr1 and Pitx2 in the LPM starts at stage 20 and stage 25, respectively (Lohr et al., 1997; Campione et al., 1999). Therefore, in temporal terms, Zic3 is expressed between Vgl/activin and Xnr1/Pitx2.

Based on these results, we propose the cascade shown in Fig. 7. Zic3 receives a signal from the initial L-R coordinator which may be an activin-like factor (Vgl, activin), and transfers this to the L-R deterministic factors, such as Pitx2, in cooperation with other asymmetrically expressed factors. The regulatory cascade may lie in a crucial part of the left-side signaling cascade.

**Fig. 7.** A hypothetical model for the involvement of Zic3 in the L-R axis formation in Xenopus. Zic3 mediates the left-sided signaling pathway. Activation of the activin-like signaling pathway on the left side induces Zic3, and Zic3 specifies the left identity by the induction of Xnr1 and Pitx2.

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**Significance of the spatial distribution of Zic3 to L-R asymmetry**

While the overexpression experiment indicated that Zic3 is involved in the development of L-R asymmetry, we found no asymmetric expression of Zic3. This could be because the Zic3 protein is asymmetrically modified or processed, and qualitatively different Zic3 proteins are therefore distributed asymmetrically. Otherwise, if a homogenous Zic3 protein is distributed symmetrically, we have to postulate the regulation of its function by other asymmetrically expressed or functioning molecules. In this respect, the hypothetical L-R gradient of small molecules that can pass through the gap junction (Levin and Mercola, 1998, 1999) would be a potential explanation.

We can not rule out the possibility that there is an L-R difference in the levels of Zic3 transcripts, which would not be detected by whole-mount in situ hybridization. Generally, asymmetrically expressed genes such as sonic hedgehog (Shh), Fgf8, activin β and activin receptor IIA, near the node/organizer have been described in chick, but not in Xenopus and mouse (Capdevila et al., 2000). Some unknown structural features might make it difficult to detect the asymmetrical expression in the latter species.

Previous studies have shown that the organizer/node plays critical roles in the development of the L-R axis (Danos and Yost, 1996; Nascone and Mercola, 1997; Lohr et al., 1997). The modification of organizer function by UV irradiation or misexpression of Xwnt8 leads to randomization of heart laterality in the Xenopus embryo (Danos and Yost, 1995). Since Zic3 is expressed in the organizer region, it may play a role in L-R specification by supporting an organizer function. In this respect, the roles of left/right dynein, inversin, Kif3a and Kif3b are interesting because mice carrying mutations in these genes show impaired motile nodal cilia, which generate a leftward flow to produce a gradient in the node (Okada et al., 1999; Takeda et al., 1999; Nonaka et al., 1998). If a similar mechanism underlies Xenopus L-R development, Zic3 may regulate or induce expression of these molecules in the organizer region to establish the asymmetric expression of Xnr1 and Pitx2.

**Deletion mutants of Zic3 are sufficient to affect the L-R laterality**

Although the precise role of the Zic3 protein has not been clarified, our structure-function study revealed that the zinc finger domain (XZ3d4, XZ3d7) or N-terminal domain (XZ3d6) is sufficient to affect the L-R laterality. Moreover, the N-terminal domain affects the laterality more efficiently on the left side, in contrast to the zinc-finger domain. We therefore think that at least two kinds of Zic3-interacting molecules are involved in the L-R signaling cascade.

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**Table 4. Zic3 determines the laterality of Pitx2 expression at early gastrula stage**

<table>
<thead>
<tr>
<th>Zic3-GR Stage 9</th>
<th>Zic3-GR Stage 10.5</th>
<th>Zic3-GR Stage 12</th>
<th>Zic3-GR Stage 14</th>
<th>Zic3-GR No dexamethasone</th>
<th>Dexamethasone only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>19</td>
<td>25</td>
<td>35</td>
<td>41</td>
<td>22</td>
</tr>
<tr>
<td>Right</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bilateral</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>% Altered expression</td>
<td>30</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>
We have previously shown that the zinc-finger domain of the Zic family can bind to the target DNA sequence of Gli (Aruga et al., 1994). The Gli proteins have a similar zinc-finger domain to the Zic family (Aruga et al., 1994; Nakata et al., 1998). Therefore, Zic3 may interact with the target DNA sequence through this domain to modify Gli protein function. However, overexpression of GLI1 did not affect the L-R laterality in the Xenopus embryo. It is possible that other Gli family proteins are involved in the L-R asymmetry, because Shh signal, which the Gli proteins are considered to mediate (Lee et al., 1997; Ruiz i Altaba, 1998), plays a role in L-R asymmetry in several species. Further investigation of the role of Gli proteins in the development of the L-R axis is required.

Another Zic3-interacting factor may bind to the N-terminal domain of Zic3. Since X23d6 has a dominant-negative function against the Zic3, the N-terminal protein may compete with endogenous Zic3 for the presumptive Zic3-interacting factor. Five distinct mutations in the zinc-finger domain of ZIC3 cause heterotaxy (Gebbia et al., 1997), whereas no individuals with a null mutation of ZIC3 have been found. The mutated genes in individuals with heterotaxy may produce an intact N-terminal domain, yet the protein can disturb the laterality of the human embryo.

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