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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

(Lohr et al., 1998; Toyoizumi et al., 2000). Injection of activin protein into the right side of the neurula disturbs the L-R laterality. Although both the early and late activin-like signals play a crucial role in establishment of the L-R asymmetry, the mechanism and molecules connecting them are still unknown.

Recently, it has been reported that mutations in human *ZIC3* cause heterotaxia (Gebbia et al., 1997). *ZIC3* is a member of the Zic family (Aruga et al., 1994, 1996a,b) of genes, which encode zinc-finger proteins and are expressed in multiple tissues during development (Yokota et al., 1996; Nagai et al., 1997). Individuals with mutated *ZIC3* suffer from congenital heart disease, caused by cardiac malformation, and show alterations in visceral situs (Casey et al., 1993). While these findings are intriguing in terms of how the L-R axis is specified, they have not revealed the role of *ZIC3* in the establishment of L-R asymmetry.

We previously demonstrated a role for *Xenopus Zic3* in the development of ectodermal tissue (Nakata et al., 1997). *Zic3* is expressed in the prospective neuroectoderm during gastrulation and induces the formation of neural and neural crest tissue. Although *Zic3* expression was also detected in the mesodermal component, the role of *Zic3* in the development of mesodermal tissue has not been clarified.

In the present study, we have investigated the role of *Xenopus Zic3* in L-R asymmetry. The results indicate that *Zic3* participates in the left signaling pathway between the L-R coordinator and determinative factors at the early gastrula stage. Furthermore, deletion analysis showed that the zinc-finger domain or N-terminal domain of *Zic3* is sufficient to alter the L-R axis, suggesting that these domains are functionally important in L-R laterality.

MATERIALS AND METHODS

Whole-mount in situ hybridization and β-gal staining

Whole-mount in situ hybridization was performed essentially as described by Shain and Zuber (1996) with slight modification. Briefly, embryos were cut before hybridization to detect the mesodermal expression of *Zic3*, *Zic1*, *Zic2* and *brachyury* more efficiently. The proteinase K (10 μg/ml) treatment was extended to 30 minutes to enhance the signal in the LPM. Digoxigenin-labeled probes were synthesized for *Xenopus Zic3*, *Zic1*, *Zic2* (Nakata et al., 1997, 1998) *Xenopus brachyury* (Smith et al., 1991), *Xnr1* (Jones et al., 1995) and *Xenopus Pitx2* (Ryan et al., 1998). β-gal staining was carried out as described (Vize et al., 1991).

Embryo manipulation

Xenopus laevis were purchased from Hamamatsu Seibutsu Kyozai (Shizuoka, Japan). Embryos were obtained from hCG-injected adult pigmented females by in vitro fertilization. The jelly coats were removed by immersing the embryos in 1% sodium mercaptoacetate (pH 9.0) for a few minutes. Embryos were cultured in 0.1× Steinberg's solution and staged according to Nieuwkoop and Faber (1967).

Microinjection was carried out as previously described (Moon and Christian, 1989). mRNA for injection was synthesized by in vitro transcription. mRNA from *Xenopus Zic3*, *Zic1*, *Zic2*, *Xenopus activin β* (Sokol et al., 1991), human *GLI1* (Kinzler et al., 1988) or *Xenopus Zic3* deletion constructs were injected with *lacZ* mRNA into the left or right dorsal blastomere of four-cell stage embryos. Injected embryos were cultured until stage 22 or stage 25 to detect *Xnr1* or *Pitx2* expression, respectively. Misexpression of *Xnr1* and *Pitx2* was scored after determination of the injected side by β-gal staining.

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.zo.utexas.edu/). In addition, we designed the following primers for use in this study. *Xnr1*: 5′-AGTCAAGTC-CTCTGCCAACC-3′, 5′-TCAAAACAACCTCATCT-CCC-3′. *Pitx2*: 5′-CTTCAGCCTCTCTTTCCACT-3′, 5′-TCACACGGGTCTGTTT-ACT-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 trace 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

Table 1. *Zic3* injection induces reversed heart and gut laterality

	Zic3 injected		*activin*	
	L	R	R	Control
Normal	124 (16/124)	92 (18/92)	73 (9/73)	84 (3/84)
Reversed heart	0	13 (7/13)	13 (6/13)	0
Reversed gut	3	2	2	0
Reversed heart and gut	1	5	8	0
% Reversed organs	3	18	24	0

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.

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,J; 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. 2I,J; 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

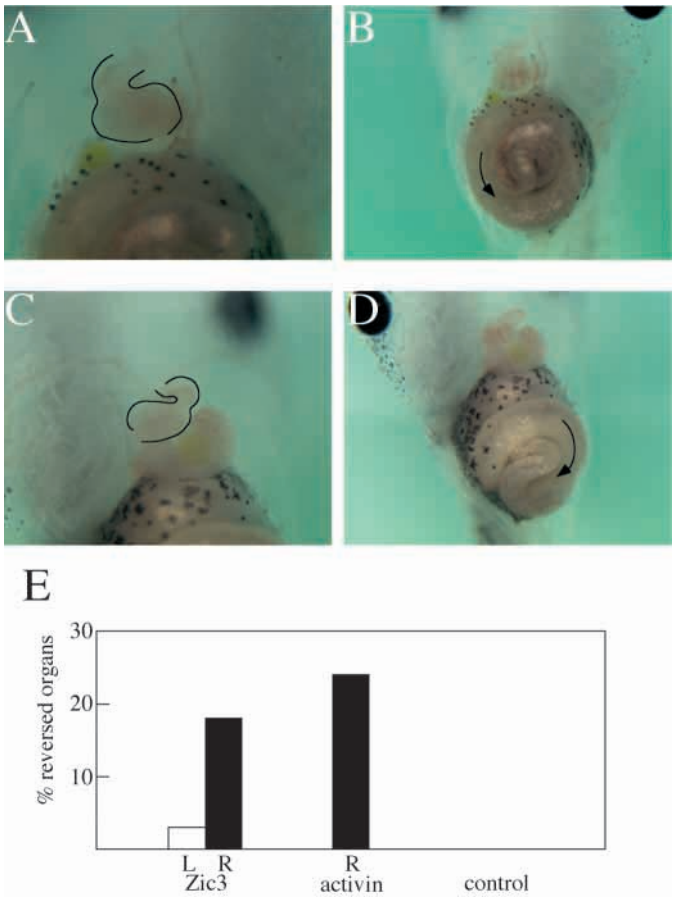

Fig. 1. Reversal of heart and gut looping in *Zic3*-injected embryos. (A,B) Wild-type *Xenopus* embryo (stage 47) with a rightward looping heart and counterclockwise coiled gut. (C,D) Embryo that was injected with 50 pg of *Zic3* on the right side, with a leftward looping heart and a clockwise coiled gut. (E) The frequency of reversed organs. Right-sided *Zic3* injection altered heart and gut looping more frequently than left-sided injection (see also Table 1). The inversion frequency by *activin* injection (0.5 pg) was determined in a positive control.

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

(A) *Xnr1* expression

	Zic3 injection		*activin* injection		*Zic1* injection		*Zic2* injection		*GLI1* injection		
	L	R	L	R	L	R	L	R	L	R	Control
Left	59	23	19	15	14	15	21	22	23	17	97
Right	2	12	0	2	1	1	1	1	0	0	0
Bilateral	19	25	0	12	4	12	2	4	1	0	4
% Altered expression	26	62	0	33	26	46	13	19	4	0	4

(B) *Pitx2* expression

	Zic3 injection		*activin* injection		*Zic1* injection		*Zic2* injection		*GLI1* injection		
	L	R	L	R	L	R	L	R	L	R	Control
Left	65	32	24	23	22	17	21	27	15	17	90
Right	1	4	0	7	0	0	0	0	0	0	0
Bilateral	2	14	0	5	0	3	0	2	0	0	0
% Altered expression	4	36	0	34	0	15	0	7	0	0	0

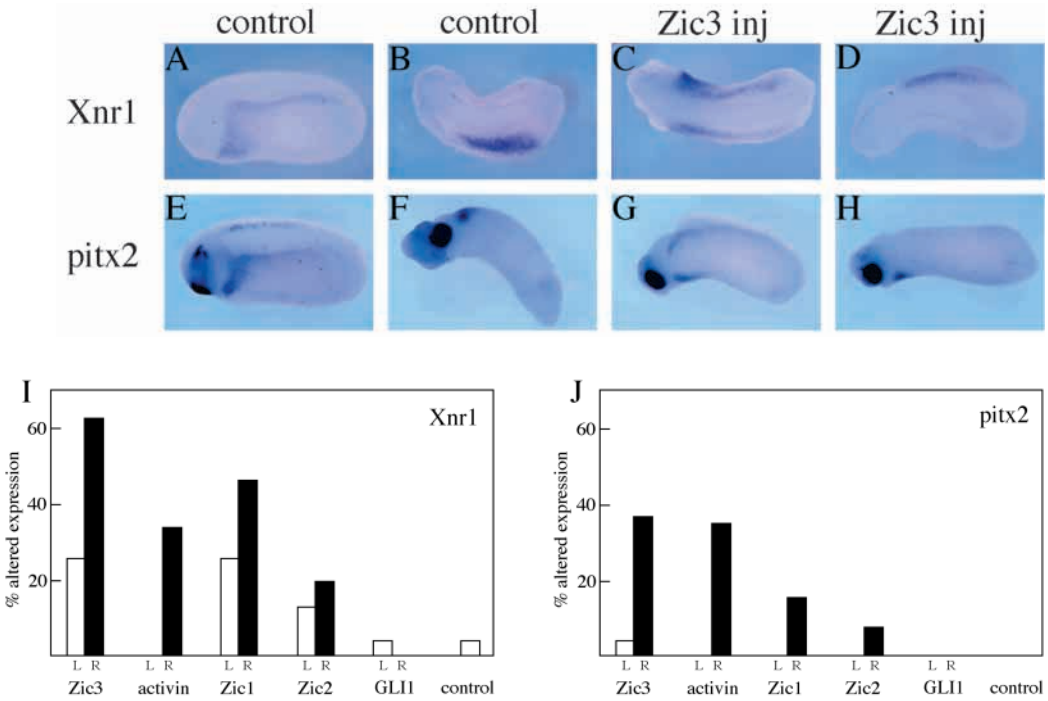

Fig. 2. Asymmetric expression of *Xnr1* and *Pitx2* is disturbed by *Zic3*. In situ hybridization of *Xnr1* (A-D) and *Pitx2* (E-H). *Xnr1* and *Pitx2* expression was observed in the left LPM (A,B,E,F). *Zic3* (100 pg)-injected embryos show bilateral (C,G) or right side (D,H) expression. The frequency of disturbed *Xnr1* (I) or *Pitx2* (J) expression by left or right side *Zic3*, *Zic1* (250 pg), *Zic2* (100 pg), *GLI1* (1000 pg) or *activin* (0.5 pg) injection.

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 an 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*

(A) *Xnr1* expression

	Zic3 injection		XZ3d4 injection		XZ3d6 injection		XZ3d7 injection		Zic3+XZ3d6 injection		
	L	R	L	R	L	R	L	R	L	R	Control
Left	59	23	57	49	20	44	21	12	18	10	97
Right	2	12	0	8	6	1	0	2	1	1	0
Bilateral	19	25	8	25	20	17	1	4	7	6	4
% altered expression	26	62	14	40	57	29	5	33	31	41	4

(B) *Pitx2* expression

	Zic3 injection		XZ3d4 injection		XZ3d6 injection		XZ3d7 injection		Zic3+XZ3d6 injection		
	L	R	L	R	L	R	L	R	L	R	Control
Left	65	32	25	27	38	40	16	12	13	8	90
Right	1	4	1	4	0	0	1	1	0	0	0
Bilateral	2	14	1	9	0	0	1	9	0	2	0
% Altered expression	4	36	7	33	0	0	11	45	0	20	0

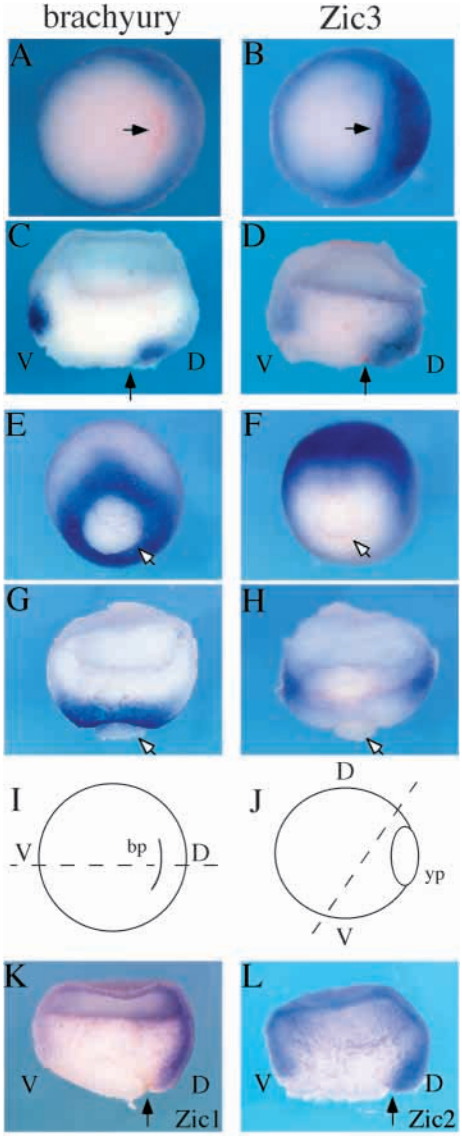

Fig. 3. *Zic3* is expressed in the mesodermal tissues at early gastrula. *Zic3* is expressed in the ring of involuting mesoderm (B,D) like the pan-mesodermal marker, *brachyury* (A,C) at stage 10.5. Arrows indicate blastopore. In C,D, embryos were cut along the broken line shown in (I). At stage 12, *Zic3* is not detected in involuting mesoderm (F,H) in contrast to *brachyury* (E,G). White arrows indicate yolk plug. (G,H) Embryos were cut along the broken line in (J). *Zic1* (K) and *Zic2* (L) are also expressed in the mesodermal tissues at early gastrula. Arrows indicate blastopore. bp, blastopore; D, dorsal side; V, ventral side; yp, yolk plug.

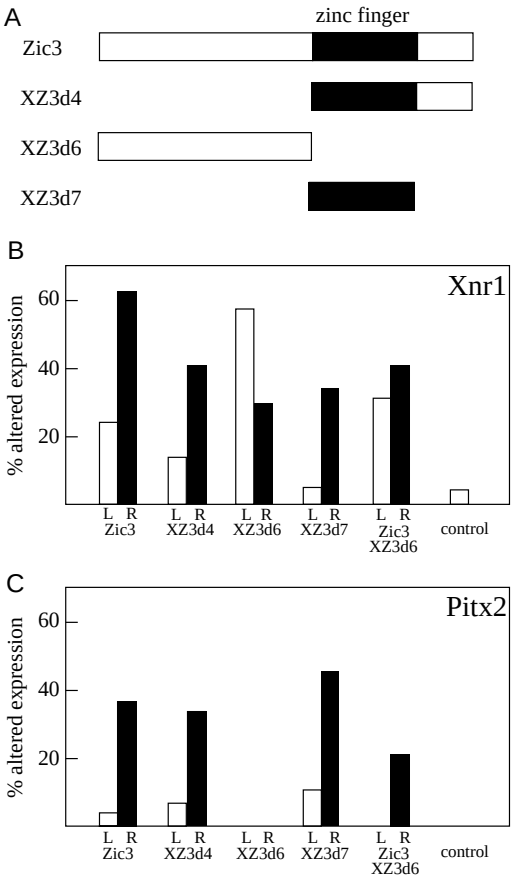

Fig. 4. Zinc-finger domain or N-terminal domain of *Zic3* alone can disturb the L-R axis. (A) Deletion constructs of *Zic3* used in this experiment. *Xnr1* or *Pitx2* expression in *Zic3*- (100 pg), XZ3d4- (500 pg), XZ3d6- (1000 pg) or XZ3d7- (500 pg) injected embryos. The frequency of the disturbed *Xnr1* (B) or *Pitx2* (C) expression by the left or right side injections.

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

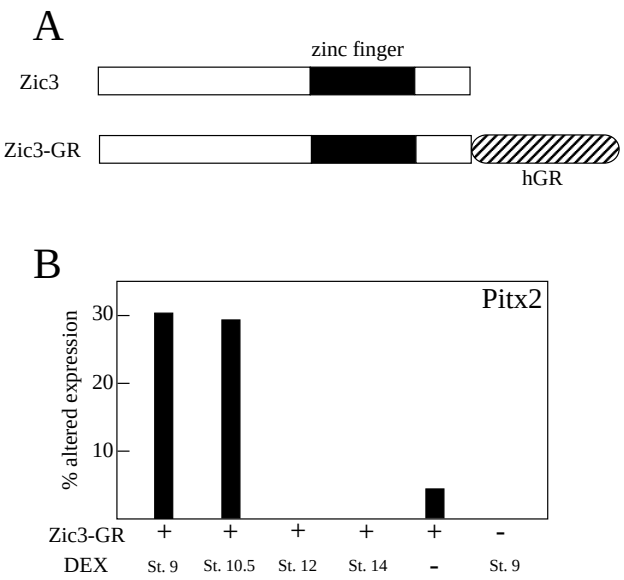

Fig. 5. *Zic3* specifies the L-R laterality at early gastrula stage. (A) Hormone-inducible construct of *Zic3* used in this experiment. (B) Disturbed expression of *Pitx2* in Zic3-GR (100 pg) injected embryo when dexamethasone was added at several stages. The frequency of the disturbed *Pitx2* expression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

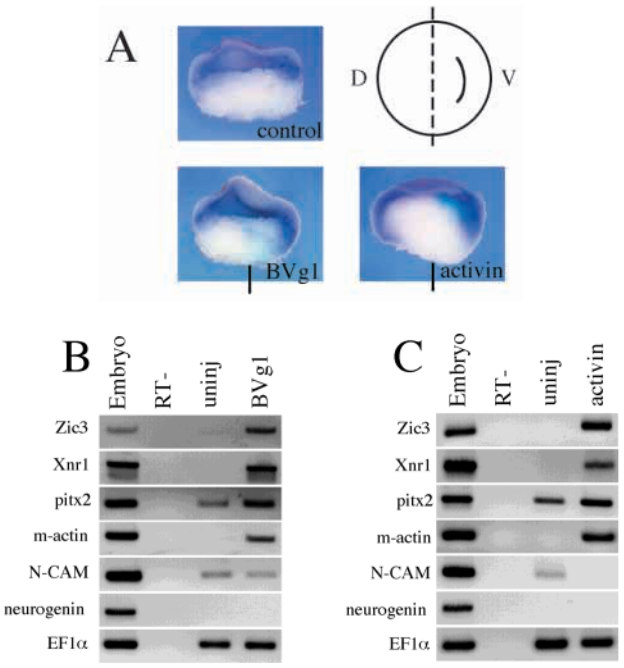

Fig. 6. *Vg1* and *activin* can induce *Zic3* in the mesoderm. (A) *Zic3* is expressed symmetrically in wild-type embryos. *Zic3* expression was enhanced in the mesoderm at stage 10.5 when *Vg1* (1000 pg) or *activin* (5 pg) was injected into the lateral side of one blastomere at the two-cell stage. Embryos were cut along the broken line shown. RT-PCR analysis of animal cap explants from embryos injected with *Vg1* (250 pg) (B) or *activin* (1 pg) (C) at stage 25. *Zic3*, *Xnr1* and *Pitx2* are induced by both genes. *Vg1* or *activin* induce the mesodermal marker, *m-actin*, but not the neural markers, *neural cell-adhesion molecule* (N-CAM) and *neurogenin*. *EF1α* was used to monitor RNA recovery.

embryos (Nakata et al., 1997), this Zic3-GR hormone-inducible system was considered to be effective.

Zic3-GR was injected into the right side of the embryo, and dexamethasone was added at various stages to analyze when *Zic3* is involved in L-R laterality (Fig. 5B; Table 4). Zic3-GR disturbed the expression of *Pitx2* on the left side when dexamethasone was added at late blastula or early gastrula stage (stage 9, 10.5) but not at late gastrula stage or later (stage 12, 14), indicating that *Zic3* specifies L-R laterality at the early gastrula stage.

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*, *NR1*, *ActRIIa* and *activin β* 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

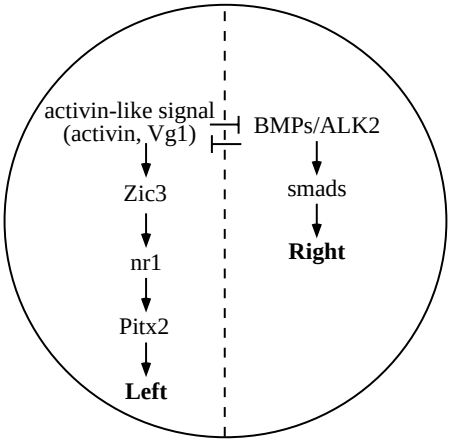

Fig. 7. A hypothetical model for the involvement of *Zic3* in the L-R axis formation in *Xenopus*. *Zic3* mediates the left-sided signaling pathway. Activation of the *activin*-like signaling pathway on the left side induces *Zic3*, and *Zic3* specifies the left identity by the induction of *Xnr1* and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

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 *Vg1*/*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 *Vg1* 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 *Vg1*/*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 (*Vg1*, *activin*), and transfers this to the L-R determinative 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.

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.

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

	Zic3-GR Stage 9	Zic3-GR Stage 10.5	Zic3-GR Stage 12	Zic3-GR Stage 14	Zic3-GR No dexamethasone	Dexamethasone only
Left	19	25	35	41	22	41
Right	4	4	0	0	0	0
Bilateral	4	6	0	0	1	0
% Altered expression	30	29	0	0	4	0

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 XZ3d6 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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