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Original Article

Xenopus neurula left-right asymmetry is respeficied by microinjecting TGF-β5 protein

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ABSTRACT A variety of TGF-β-related ligands regulate the left-right asymmetry of vertebrates but the involvement of TGF-βs in left-right specification has not been reported. We assessed whether TGF-β signaling is involved in the left-right specification of *Xenopus* post-gastrula embryos by microinjecting *Xenopus* TGF-β5 protein into the left or right flank of neurula–tailbud embryos. Injection on the right side of neurulae caused left-right reversal of the internal organs in 93% of the embryos, while injection on the left side caused less than 5% left-right reversal. Expression of *Xenopus nodal related-1* (*Xnr-1*), *Xenopus antivin* and *Xenopus Pitx2,* which are normally expressed on the left, was unaltered by the left-side injection. In contrast, right-side injection into neurulae induced the expression of these genes predominantly on the right side. Right-side injection into tailbud embryos caused bilateral expression of these handed genes. Time course analysis of asymmetric gene expression revealed that *Xnr-1* could be induced by TGF-β5 at late neurula stage, while *antivin* and *Pitx2* could be induced by TGF-β5 at the later tailbud stage. Injection of the antisense morpholino oligonucleotide against *Xenopus TGF-β5* into the left dorsal blastomere inhibited the normal left-handed expression of *Xnr-1* and *Pitx2,* and caused the organ reversal in the injected embryos. These results suggest that normal left-right balance of endogenous TGF-β5 signaling in the neurula embryo may be needed to determine the laterality of the asymmetric genes and to generate the correct left-right axis.

KEY WORDS: *TGF-β5, Xnr-1, antivin, Pitx2, morpholino*

Introduction

To better understand the mechanisms that establish the body plan of vertebrates, we must identify the signaling molecules and the molecular networks that are involved in the formation of the antero-posterior, dorso-ventral and left-right axes. Molecular mechanisms controlling the left-right asymmetry had long been unknown, but recent studies identified various genes involved in this process. These studies showed that members of the transforming growth factor(TGF)-β superfamily play a pivotal role in the establishment of left-right asymmetry.

Left-right asymmetry is established by the asymmetric expression of *nodal* gene coding a member of TGF-β superfamily in vertebrate embryos (Burdine and Schier, 2000; Capdevila *et al.*, 2000). The left-handed expression of *nodal* is conserved in all the vertebrate species examined (Levin *et al.*, 1995; Lowe *et al.*, 1996; Rebagliati *et al.*, 1998). The genetic cascades leading to such asymmetric *nodal* expression are best understood in the chick embryo. Initially, *activin βB*, also a member of the TGF-β superfamily, and *activin type IIA receptor* for activin signaling are expressed in the right side of the primitive streak and Hensen's node (Levin *et al.*, 1995; Stern *et al.*, 1995; Levin *et al.*, 1997), under the control of the gap junctional communication (Levin and Mercola, 1999). Subsequently, they activate the right-sided expression of *bone morphogenetic protein-4* (*BMP-4*) (Rodriguez-Esteban *et al.*, 1999) and elicit the left-sided expression of *Sonic hedgehog* (*Shh*) (Monsoro-Burq and Le Douarin, 2001). *BMP type IA receptor* and *Smad1,* which are involved in the BMP signal transduction pathway, are also predominantly expressed in the right side of the node (Monsoro-Burq and Le Douarin, 2000, 2001). The expression and the interaction of the *Shh* and *BMP* products eventually induces the left-handed expression of *chick nodal related-1* (*Cnr-1*) in the node and the lateral plate mesoderm (LPM) at the somite stage (Piedra and Ros, 2002; Schlange *et al.*, 2002). In the mouse embryo, left-handed *nodal* induces the expression of *lefty-2* in the left LPM. The *lefty-2* product then acts as a feedback inhibitor of *nodal* expres-

Abbreviations used in this paper: LPM, lateral plate mesoderm; MO, Morpholino oligonucleotide; TGF-β5, Transforming Growth Factor-β5; Xnr-1, Xenopus nodal related-1; Xatv, Xenopus antivin.

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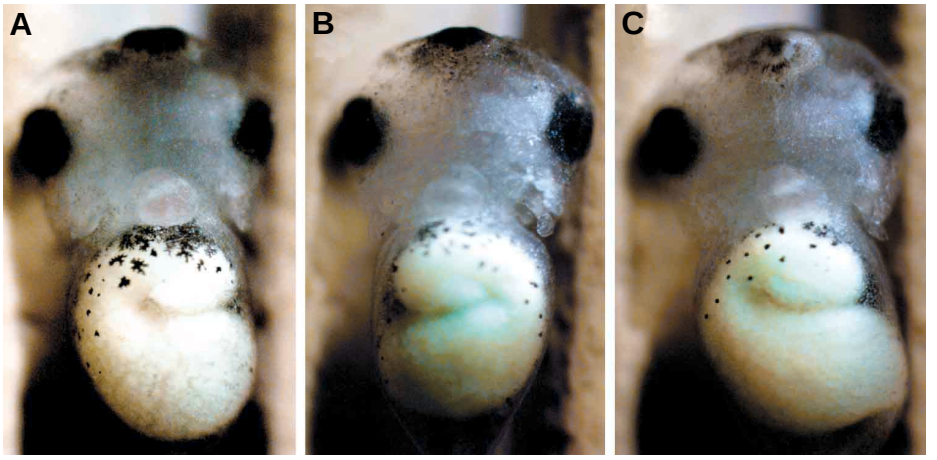


Fig. 1. Effect of TGF-β5 protein injection on the left-right asymmetry of *Xenopus* internal organs. *All embryos are viewed from the ventral side.* **(A)** *An uninjected sibling embryo at stage 42 shows normal heart looping, and the ventral pancreas is situated in the left side.* **(B)** *An embryo after injecting 10 pg TGF-β5 in the right flank at the mid-neurula stage (stage 15). Both heart and visceral organs are inverted.* **(C)** *An embryo after injecting 10 pg TGF-β5 in the left flank at stage 15. Left-right orientation of the internal organs is normal.*

sion by down-regulating the autoregulatory loop of *nodal* (Meno *et al.*, 1999; Meno *et al.*, 2001; Hamada *et al.*, 2002). *Lefty* also belongs to the TGF-β superfamily, and the left-handed expression of *lefty* is conserved in all the vertebrates examined, which is the same as *nodal* (Meno *et al.*, 1996; Meno *et al.*, 1997;Thisse and Thisse, 1999; Branford *et al.*, 2000; Cheng *et al.*, 2000; Ishimaru *et al.*, 2000; Tanegashima *et al.*, 2000). *Nodal* subsequently induces the expression of *Pitx2* in the left LPM. *Pitx2* maintains its asymmetric expression during the organogenetic stages, and the left-sided *Pitx2* expression is also conserved among the vertebrates (Logan *et al.*, 1998; Piedra *et al.*, 1998; Ryan *et al.*, 1998; Yoshioka *et al.*, 1998; Campione *et al.*, 1999; Schweickert *et al.*, 2000).

Recently, GDF(growth differentiation factor)-1 has been recognized as the TGF-β superfamily ligand indispensable for the establishment of left-right asymmetry in mouse embryos, though GDF-1 expression is bilateral. Mice lacking *GDF-1* was reported to show randomization of left-right axis (Rankin *et al.*, 2000), probably under the control of the hedgehog signaling (Znang *et al.*, 2001). *GDF-1* is highly homologous to *Xenopus Vg1* (Lee, 1990). When *Vg1* mRNA is injected into the right blastomeres at the 16-32 cell stage, the mRNA fully inverts the left-right axis of *Xenopus* embryos (Hyatt *et al.*, 1996; Hyatt and Yost, 1998). Cross-species experiments revealed that injection of mouse *GDF-1* mRNA also alters the laterality of *Xenopus* internal organs (Wall *et al.*, 2000). Thus, various TGF-β superfamily ligands have been identified as key players of left-right signaling, and their relationships have been reported by early investigators.

Although many studies revealed that various ligands belonging to TGF-β superfamily are essential for the left-right signaling pathway, we do not yet know whether TGF-β, a core member of the superfamily, participates in the establishment of left-right asymmetry. In this study, we tested this idea using *Xenopus laevis* embryos.

TGF-βs act as regulators of cell proliferation, differentiation and transformation of cancer cells (Todaro *et al.*, 1981; Todaro, 1982; Roberts *et al.*, 1990; Kingsley, 1994). Furthermore, in the early chick embryo, *TGF-β2* and *TGF-β3* are expressed in the presumptive heart region, and it has been suggested that both TGF-β2 and TGF-β3 are involved in the formation of the heart tube (Potts *et al.*, 1992; Jakowlew *et al.*, 1994; Nakajima *et al.*, 1998; Boyer *et al.*, 1999; Yamagishi *et al.*, 1999). In addition, knockout mice of *TGF-β2* suffer abnormalities of the heart, lung, skeleton, urinary organs, sensory organs, and reproductive organs (Sanford *et al.*, 1997). Moreover, mutation of the *TGF-β type II receptor* causes carcinogenesis in the digestive organs (Markowitz *et al.*, 1995), and mice lacking this gene are embryonic lethal at 10.5 dpc due to the aplasia of the circulatory system (Oshima *et al.*, 1996). Such observations suggest that the TGF-β signaling pathway may be important in the morphogenesis of the left-right asymmetric organs.

Though *Xenopus laevis* embryo is a useful model organism for molecular biology, the early molecular events for left-right specification in *Xenopus* embryos are much less understood in comparison to chick and mouse embryos. It is likely, however, that TGF-βs, particularly endogenous *Xenopus* TGF-β5, may participate in the left-right specification in the *Xenopus* neurulae because *Xenopus* TGF-β5 starts to be expressed as a zygotic gene in the dorsal organizer region of the late gastrula stage (stage 12/13), after

TABLE 1

INCIDENCE OF THE LEFT-RIGHT REVERSAL OF HEART AND VISCERAL ORGANS CAUSED BY RIGHT-SIDE INJECTION OF 50 PG TGF-β5 PROTEIN INTO NEURULA–TAILBUD EMBRYOS

Stage	Total incidence of L-R inversion	L-R reversal of both heart and gut	L-R reversal of heart-alone	L-R reversal of gut-alone (%)
13-14	94	88	5	1
	128/136	119/136	7/136	2/136
15-16	94	78	5	11
	51/54	42/54	3/54	6/54
17-18	90	63	15	12
	97/108	68/108	16/108	13/108
19-20	79	45	24	10
	104/131	59/131	32/131	13/131
21-22	75	38	30	7
	124/166	63/166	50/166	11/166
23-24	45	9	36	0
	88/195	17/195	71/195	0/195
25-26	34	5	28	1
	57/168	9/168	47/168	1/168
27-28	0	0	0	0
	0/49	0/49	0/49	0/49
29-30	0	0	0	0
	0/45	0/45	0/45	0/45
31-32	4	0	4	0
	3/68	0/68	3/68	0/68
33-34	0	0	0	0
	0/24	0/24	0/24	0/24

The top numbers show the incidence of left-right reversal in %, the bottom numbers show the ratio of *embryos with organ reversal/survived embryos*. The injection into the neurulae resulted in embryos with left-right inversion of both their heart and gut. In contrast, the injection into the tailbud embryos resulted in embryos with left-right inversion of the heart only.

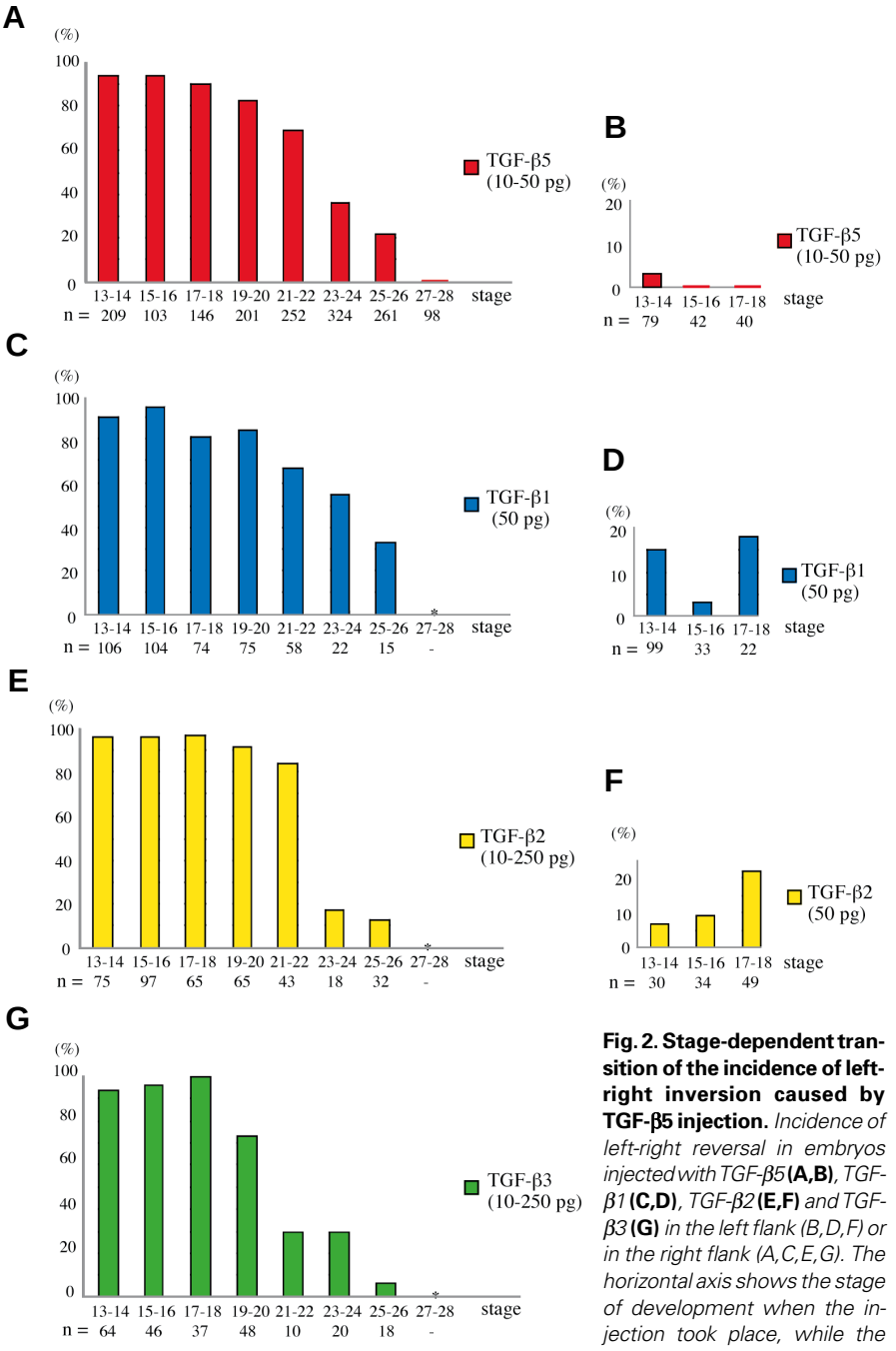


Fig. 2. Stage-dependent transition of the incidence of left-right inversion caused by TGF-β5 injection. *Incidence of left-right reversal in embryos injected with TGF-β5* **(A,B)**, *TGF-β1* **(C,D)**, *TGF-β2* **(E,F)** *and TGF-β3* **(G)** *in the left flank (B,D,F) or in the right flank (A,C,E,G). The horizontal axis shows the stage of development when the injection took place, while the longitudinal axis represents the ratio of left-right inversion as percent. Asterisk (*) shows the case not tested. n, number of embryos that reached stage 41-42 and were checked for left-right orientation.*

which it is expressed in the paraxial mesoderm in juxtaposition to LPM, the field of handed gene expression (Kondaiah *et al.*, 2000). Furthermore, as mentioned, TGF-β is involved in the organogenesis of the asymmetric heart and digestive organs in higher vertebrate embryos, and the TGF-β signaling pathway shares some intracellular components with the Nodal and Activin pathways that show asymmetric expression patterns in the chick embryo. TGF-β5 displays 76%, 66%, 69% homology to human TGF-β1, β2, β3, respectively in the C-terminal mature peptide (Kondaiah *et al.*, 1990). However, little is known about the function of TGF-β5 in the early development of *Xenopus* embryos.

Here we report the effects of microinjecting *Xenopus* neurulae or tailbud embryos on either side with *Xenopus* TGF-β5 protein. We found that injection of this protein on the right flank remarkably inverted the left-right orientation of the visceral organs and seriously altered the laterality of the left-handed genes. Conversely, injection of morpholino oligonucleotide directed against *TGF-β5* on the left side clearly downregulated the expression of left-handed expression of *Xnr-1* and *Pitx2*, and caused left-right reversal. These observations suggest that the establishment of normal left-right asymmetry depends on the correct left-right balance of TGF-β-dependent signaling.

Results

Injection of TGF-β5 Protein into the Right Side of Neurulae results in Left-Right Inversion of the Heart and Visceral Organs in almost All Injected Embryos

Remarkably, right-side injection of TGF-β5 protein into the neurulae caused the complete reversal of the left-right axis. By the injection at stage 13-18, 93% (n=425/458) of the embryos showed left-right reversal of their internal organs (Figs. 1B, 2A). In contrast, left-side injection of TGF-β5 into neurulae did not significantly affect the laterality of the internal organs (Figs. 1C, 2B). The incidence of left-right reversal in stage 13-18 left-injected embryos was 1% (n=2/161, Fig. 2B). TGF-β5 injection did not affect the morphology of the heart and visceral organs apart from their altered left-right orientation. Furthermore, the injected embryos grew up to be normal larvae (DAI=5, Kao and Elinson, 1988). After the injection, 80% (n=458/571) of the embryos survived for the 4 days of subsequent cultivation. When stage 21-26 tailbud embryos were injected on the right with TGF-β5, left-right reversal of the internal organs occurred in 42% of the embryos (n=350/837, Fig. 2A). However, altered orientation of the heart or visceral organs was negligible when stage 27-34 late tailbud embryos were injected on the right with TGF-β5 (n=3/186, Table 1).

Right-injection of TGF-β5 into stage 13-18 neurulae most frequently caused left-right inversion of both the heart and gut (Table 1), but this was reduced in embryos injected at stage 19-22, which instead more often had inversion of the heart only. For tailbud embryos, TGF-β5 injection more frequently caused heart reversal than gut reversal. For example, 45% of the embryos injected at stage 23-24 showed heart reversal while only 9% showed gut reversal.

As a control experiment, we injected 5 nano-liter of the 1% BSA solution lacking the growth factor into neurulae. In almost all cases,

regardless of the injection side, the orientation of the internal organs was normal [right-side injection, 0% (n=0/68); left-side injection, 2% (n=1/42)]. The ratio of spontaneous left-right reversal in uninjected sibling embryos was 2.4% (n=15/620).

TGF-β1, TGF-β2 and TGF-β3 have very similar structures to *Xenopus* TGF-β5 (Kondaiah *et al.*, 1990). Neurula embryos injected with either one of TGF-β1, TGF-β2 or TGF-β3 showed more than 90% reversal of the internal organs (Fig. 2 C,E,G). We found that the left-right reversal caused by right-side injection of TGF-β1 into mid-neurulae was dependent on the dose of TGF-β1 (Table 2). Thus, TGF-βs have a powerful potential to invert the left-right orientation of organs when injected on the right at the neurula stage and, to a lesser extent, at the tailbud embryo stage.

Injection of Other Growth Factors into Neurulae has Little Effect on the Left-Right Orientation of the Internal Organs

We examined whether the left-right orientation could be inverted by injecting early-mid neurulae with growth factors other than TGF-βs (Table 3). The following proteins were injected on either side of the embryos; FGF-8 (250-500 pg, n=141), FGF-10 (500 pg, n=177), IGF-I (500 pg, n=145), IGF-II (500 pg, n=119), PDGF-AA (500 pg, n=90), VEGF (500 pg, n=85), GDF-6 (500-1250 pg, n=237) or BMP-3 (500-1000 pg, n=245). None of these factors affected the left-right orientation of the internal organs.

Laterality of Xnr-1, Xatv *and* Pitx2 *Expression is affected by TGF-β5 Injection*

It is known that *Xnr-1* begins to be expressed in the left LPM at the neural tube stage (stage 19), and its expression continues at least until stage 26 (Lowe *et al.*, 1996). *Xatv* (*Xlefty-b*) begins to be expressed in the left LPM from the tailbud stage (stage 23) (Cheng *et al.*, 2000; Branford *et al.*, 2000), as *Pitx2* does (stage 24/25) (Ryan *et al.*, 1998; Campione *et al.*, 1999). We investigated whether the expression patterns of *Xnr-1*, *Xatv* and *Pitx2* are altered by TGF-β5 injection. When neurulae were injected on the left, the location of *Xnr-1*, *Xatv* and *Pitx2* expression was unaltered in almost all the injected embryos (Figs. 3 A,D,F and 5A), although a few completely lacked *Xnr-1*, *Xatv* and *Pitx2* expression. On the other hand, the right-side injection at the same dose dramatically changed the laterality of these genes from the left LPM to the right LPM, both LPMs, or neither LPM in the majority of the embryos (Figs. 3 B-C, E,G, and 5B). Above all, right-only expression was a dominant pattern of all the three genes.

When early tailbud embryos were injected on the right with TGF-β5, most of the embryos showed bilateral *Xnr-1* expression (48%, n=26/54) and bilateral *Pitx2* expression (67%, n=38/57) (Figs. 4 A,C, and 5C). That is, neurula stage injection induced 'right-alone expression' of *Xnr-1* and *Pitx2* more effectively than early tailbud stage injection. In the case of *Xatv*, after TGF-β5 injection at stage 21-22, *Xatv* showed various expression patterns: 38% (n=19/50) of the embryos showed right-alone expression, 26% (n=13/50) showed bilateral expression, 20% (n=10/50) lacked the expression and

TABLE 2

DOSE-DEPENDENT CHANGE OF THE INCIDENCE OF LEFT-RIGHT REVERSAL IN EMBRYOS INJECTED WITH TGF-β1 LIGAND INTO THE RIGHT FLANK

Dose (pg)	Total number of the injected embryos	Survival ratio of the injected embryos	Incidence of L-R inverted embryos
50	N=121	86	95
		104/121	99/104
10	N=69	91	95
		63/69	60/63
5	N=66	70	67
		46/66	31/46
1	N=69	65	60
		45/69	27/45
0,5	N=48	100	40
		48/48	19/48
0,05	N=43	100	9
		43/43	4/43
0	N=20	100	0
		20/20	0/20

Injection was performed at the mid-neurula stage. Lower doses of TGF-β1 decreased the incidence of embryos with inversions.

TABLE 3

EFFECT OF INJECTING NON-TGF-β GROWTH FACTORS ON THE INCIDENCE OF THE LEFT-RIGHT REVERSAL OF HEART AND VISCERAL ORGANS

(a) FGF-8, 250-500 pg			
	stage:	13-14	15-16
right		6	14
		3/52	3/22
left		5	4
		2/43	1/24
(b) FGF-10, 500 pg			
	stage:	13-14	15-16
right		2	0
		1/43	0/48
left		3	0
		1/38	0/48
(c) IGF-I, 500 pg			
	stage:	13-14	15-16
right		0	0
		0/48	0/36
left		4	0
		1/24	0/37
(d) IGF-II, 500 pg			
	stage:	13-14	15-16
right		0	3
		0/34	1/36
left		0	0
		0/25	0/24
(e) PDGF, 500 pg			
	stage:	13-14	15-16
right		0	-
		0/45	-
left		0	-
		0/45	-
(f) VEGF, 500 pg			
	stage:	13-14	15-16
right		0	14
		0/24	3/22
left		9	0
		2/23	0/16
(g) GDF-6, 500-1250 pg			
	stage:	13-14	15-16
right		1	0
		1/92	0/48
left		0	0
		0/72	0/25
(h) BMP-3, 500-1000 pg			
	stage:	13-14	15-16
right		0	0
		0/72	0/51
left		0	0
		0/73	0/49

Injection was performed at the early–mid neurula stage. These growth factors had little or no effect on the left-right orientation of *Xenopus* internal organs.

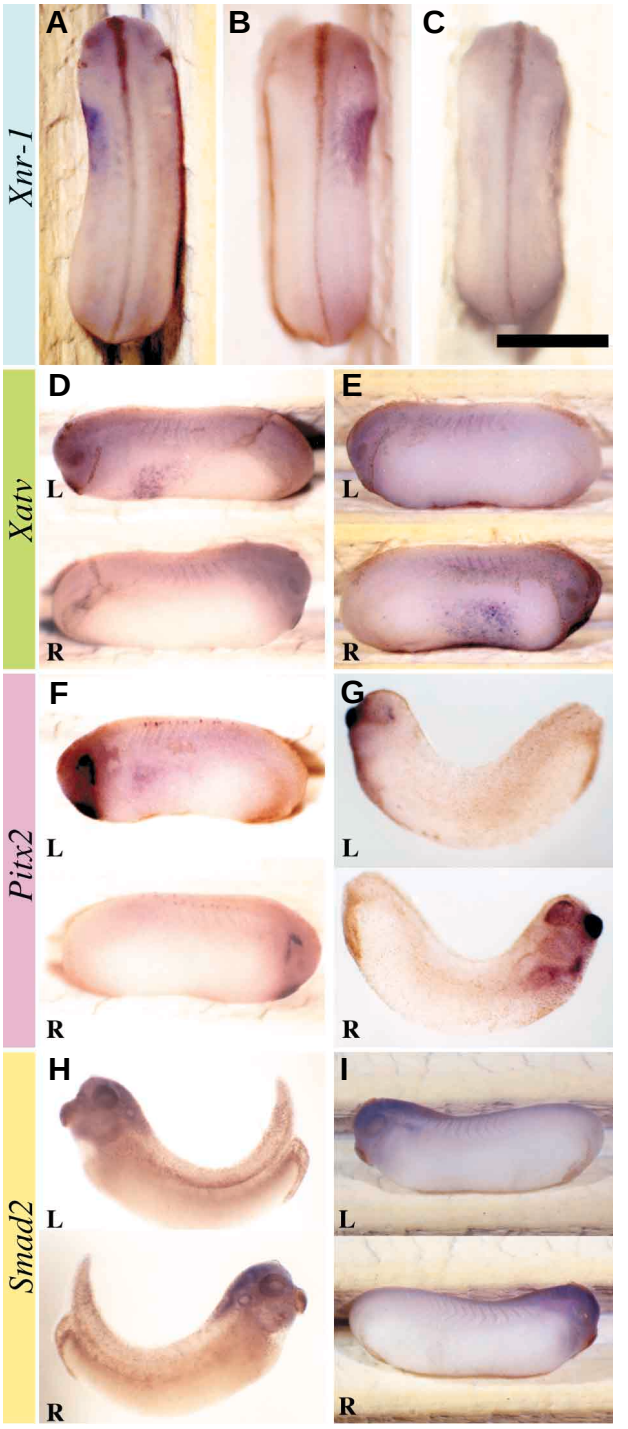


Fig. 3. Effect of injecting TGF-β5 into one side of neurula embryos on asymmetric gene expression. *TGF-β5 (50 pg) was injected at early-mid neurula stage (stage 13-16) and the embryos were fixed at the tailbud stage (stage 24-28) and assessed for* Xnr-1 *(left injection, A; right injection, B-C),* Xatv *(left injection, D; right injection, E),* Pitx2 *(left injection, F; right injection, G) and* Smad2 *(H, I) expression. After injecting in the left flank,* Xnr-1 **(A)**, Xatv **(D)** *and* Pitx2 **(F)** *were expressed on the left . After injecting in the right flank,* Xnr-1 *was expressed predominantly on the right only* **(B)**, *or less frequently on both sides or not at all* **(C)**. *After injecting in the right side,* Xatv **(E)** *and* Pitx2 **(G)** *were also expressed predominantly on the right only. The bilateral expression of* Pitx2 *around the eyes and cement gland was not altered by either the left side or right side injection.* **(H)** *Expression of* Smad2 *in the untreated embryo at stage 34. Note the absence of the mRNA in both LPMs.* **(I)** *Expression of* Smad2 *after injecting TGF-β5 in the right side.* Smad2 *was not induced in either LPM. The embryos in A-C are dorsal views with the anterior of the embryo to the top of the panels. The embryos in D-I are lateral views. Small capitals represent the left side (L) or the right side (R). Scale bar, 1 mm.*

16% (n=8/50) showed left-alone expression (Figs. 4B, 5C). *Xatv* was more effectively induced on the right side of tailbud embryos than the others.

It is known that the phosphorylated Smad2 protein transduces the intracellular signaling of TGF-β in vertebrate embryos (Nomura and Li, 1998; Labbe *et al.*, 1998; Yeo *et al.*, 1999; Dick *et al.*, 2000). We thus examined *Smad2* expression but did not detect *Smad2* mRNA in either LPM of the normal embryo (n=27, Fig. 3H) as was also previously reported by Howell *et al.* (2001). Injection of TGF-β5 into the neurulae also did not induce the ectopic expression of *Smad2* on either side (n=0/34, Fig. 3I). As Smad2 protein has been reported to be abundant in the LPM at the neurula-tailbud stages (Schohl and Fagotto, 2002), it is possible that new protein synthesis of Smad2 is not needed for left-right signaling. *Smad2* expression in anterior head and eyes was also not changed by the injection.

Altered Expression Patterns of Asymmetric Genes correlate with the Incidence of Left-Right Organ Reversal

We have thus far found that not only is there frequent reversal of internal organ orientation when TGF-β5 is injected on the right (Figs. 1,2), genes that are normally expressed asymmetrically on the left also show serious changes to their expression patterns (Figs. 3,4 and 5 A-C). We asked whether these two observations correlate. That is, do the changes in the laterality of *Xnr-1*, *Xatv* and *Pitx2* expression correlate with the reversed orientation of the internal organs? To address this issue, we compared the actual incidence of left-right organ reversal resulting from right-side injection (Table 1) to the incidence predicted on the basis of alterations in the laterality of *Xnr-1*, *Xatv* and *Pitx2* expression (Fig. 5D). To calculate the latter value, we employed several assumptions. First, expression that occurs on the left only or that predominates on the left leads to normal organ orientation. Second, expression that occurs on the right only or that predominates on the right leads to organ reversal. Third, 75% of the embryos with symmetric bilateral expression or that lack expression will develop reversed situs of either the heart or gut (Fig. 5D). The expected values of left-right inversion were then calculated using the following equation:

[expected value of left-right reversal (%)] = [incidence of right-only expression (%)] + [incidence of right-dominant expression (%)] + [incidence of symmetric expression (%)] x 3/4 + [incidence of no expression (%)] x 3/4.

We first compared the expected values with the real incidence of left-right reversal in the embryos injected on the right with TGF-β5 at the neurula stage. The incidence of left-right reversal based on the *Xnr-1*, *Xatv* or *Pitx2* expression pattern was predicted to be 80%, 84% and 91%, respectively (Fig. 5D). The actual incidence of left-right reversal by right-side TGF-β5 injection was 94% (stage 13-16 injection, n=179/190; Table 1). Thus, the predicted values and the real incidence was substantially coincidental.

We next compared the expected values with the actual incidence of left-right reversal for the embryos that had been injected

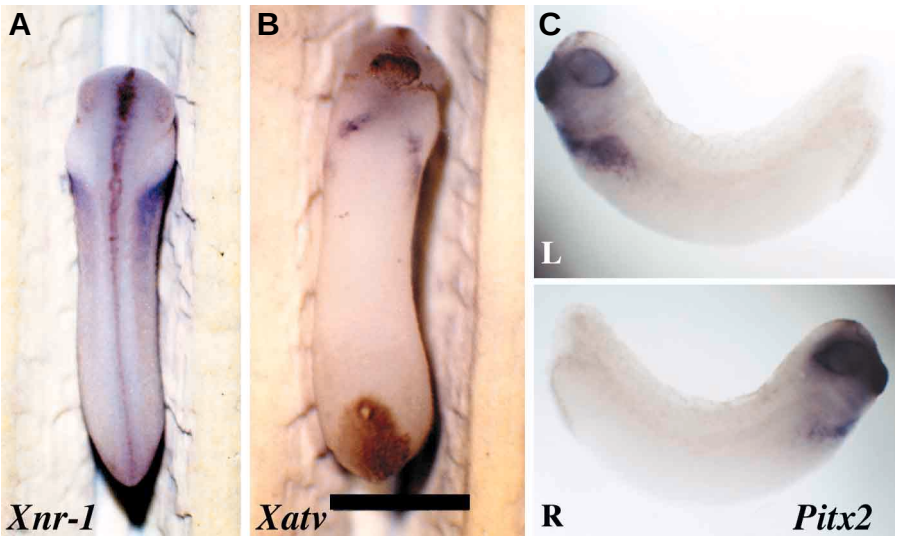


Fig. 4. Effect of injecting TGF-β5 into the right side of the tailbud embryos on asymmetric gene expression patterns. *TGF-β5 (50 pg) was injected at stage 21-24 and the embryos were fixed at stage 26-30 and assessed for the expression of* **(A)** Xnr-1 *(dorsal view of bilateral expression),* **(B)** Xatv *(ventral view of bilateral expression), and* **(C)** Pitx2 *(lateral view of bilateral expression). By injecting TGF-β5 into the right side of tailbud embryos, most of the embryos showed the bilateral expression of these genes. Scale bar, 1 mm.*

on the right in the tailbud stages (Fig. 5D). The incidence of left-right reversal was predicted to be 66% for stage 21-22 injection, based on the altered *Xnr-1* expression by the injection at the stage. Similarly, based on the *Xatv* expression pattern caused by stage 21-22 injection, we predicted that left-right reversal would be 73%. For stage 23-24 embryos, the incidence was predicted to be 37% by the altered laterality of the *Pitx2* expression.

The actual left-right reversals after right-side TGF-β5 injection at the corresponding stages were 75% by stage 21-22 injection (n=124/166) and 45% by stage 23-24 injection (n=88/195). Thus, the predicted values coincided with the real incidence for embryos injected in the tailbud stages as well as the predicted values of the neurula stage injection did. This suggests that these three genes are intimately involved in the TGF-β5-induced left-right reversal.

The Timing of Xnr-1, Xatv a*nd* Pitx2 *Expression is not altered by Injection with TGF-β5*

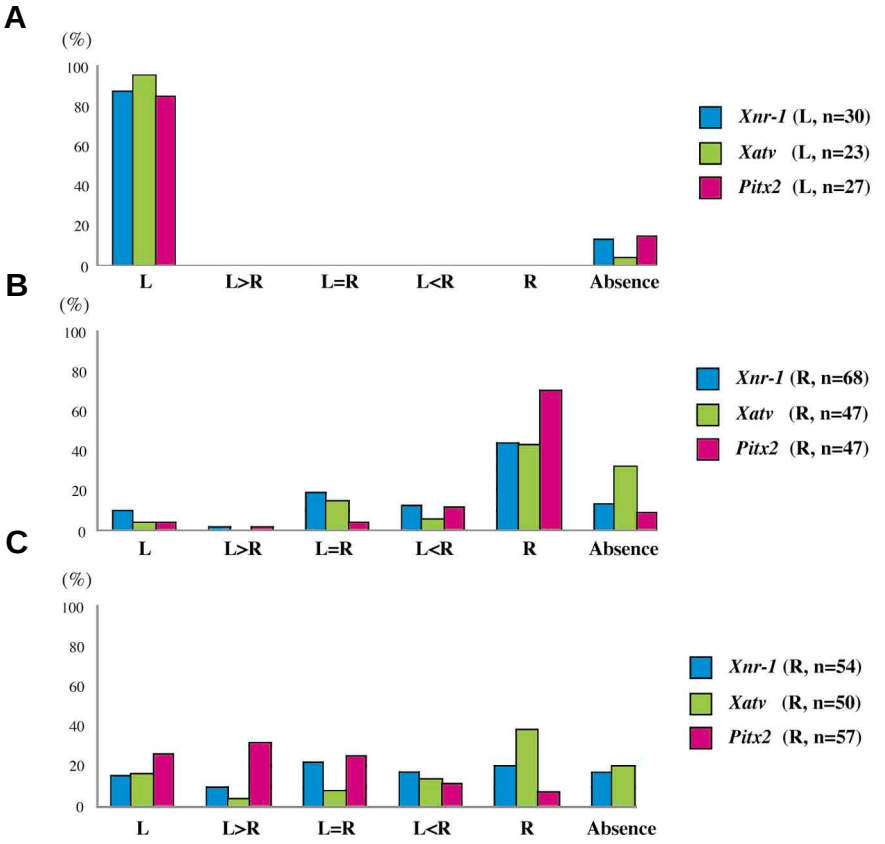
We found that laterality of the asymmetric genes was changed from left to right by the injection of TGF-β5 (Figs. 3,4 and 5). From this result we assumed that, immediately after the right-side injection, TGF-β5 induced the right-sided expression of the asymmetric genes. Supporting this idea, we reported previously that Activin A injection at the early neurula stage rapidly induced the *Xnr-1* expression in the injected LPM by the mid-neurula stage (Toyoizumi *et al.*, 2000). *Xnr-1* is normally expressed at a later neurula stage (stage 19). To test whether TGF-β5 induces the ectopic expression of the asymmetric genes, we assessed their temporal expression in injected embryos and compared this to their normal left-handed temporal expression patterns (Figs. 6,7). Thus, early neurulae (stage 13-14) were injected on either side with TGF-β5, fixed at mid-neurula (stage 15-16) or later stages and the expression of the three genes was examined. TGF-β5 injection did not induce the expression of *Xnr-1* until the late neurula stage (Figs. 6 A-D, 7A) and significant expression of *Xatv* and *Pitx2* was not observed until the early tailbud stage (Figs. 6 E,F and 7 B,C). Thus, the timing of expression was not altered by the injection.

This suggests that the injected TGF-β5 protein inverts the left-right axis by employing the normal developmental schedule. The axial expression of *Xatv* (Cheng *et al.*, 2000; Branford *et al.*, 2000) and the anterior expression of *Pitx2* (Ryan *et al.*, 1998; Campione *et al.*, 1999) were not changed by the TGF-β5 protein injection.

It is known that *Xenopus Sonic hedgehog* (*Shh*) is expressed in the midline tissues including notochord and floor plate (Ekker *et al.*, 1995), and that *Shh* is induced by the administration of activin, a member of TGF-β superfamily, in the animal cap assay (Yokota *et al.*, 1995; Takabatake *et al.*, 1996). Furthermore, *Shh* is involved in the left-right determination in chick (Levin *et al.*, 1995) and mouse (Meyers and Martin, 1999; Zhang *et al.*, 2001) embryos. We therefore assessed *Shh* expression in TGF-β5-injected neurulae and normal embryos. Normal *Shh* expression was observed in the midline tissues of the uninjected early neurula–tailbud embryos (stage 14-36) but not in either LPM during this period (n=0/74). In the TGF-β5-injected neurulae (stage 15-20), *Shh* expression in the midline tissues was not affected and the ectopic expression of *Shh* in the LPM was not observed (left-injection, n=0/20; right-injection, n=0/42; Fig. 6G).

Inhibition of Endogenous TGF-β5 Signaling in Xenopus *Embryos induces the Reversal of Left-Right Orientation*

In order to examine the involvement of endogenous TGF-β5 signaling in the left-right specification of *Xenopus* embryos, we injected 1mM of antisense TGF-β5 MO into one of the dorsal blastomeres of the 4-cell stage embryo. The antisense MO acts to inhibit local TGF-β5 signaling *in vivo*. Right-side injection of antisense TGF-β5 MO caused only 3% left-right reversal (n=2/79; Fig. 8 A,F). In contrast, left-side injection reversed the left-right orientation in 30% (n=29/97) of the injected embryos (Fig. 8 B,F). Injection of TGF-β5 MO into the ventral blastomere did not cause significant left-right reversal, probably because *TGF-β5* is expressed mainly in the dorsal structures. At the molecular level, injection of the MO into the dorsal blastomere inhibited the expression of *TGF-β5* in somites on the same side (Fig. 8 C-E). This suggests that the paraxial expression of *TGF-β5* is maintained by the autoregulatory positive feedback. Injection of the TGF-β5 MO severely affected the expression of the left-handed genes only when it was injected on the left side. Most of the embryos injected on the right with TGF-β5 MO (93%, n=43/46) still expressed *Xnr-1* in the left LPM only (Fig. 9 A-B, E). On the other hand, injection on the left strongly inhibited the left-handed *Xnr-1* expression, as only 40% (n=17/42)



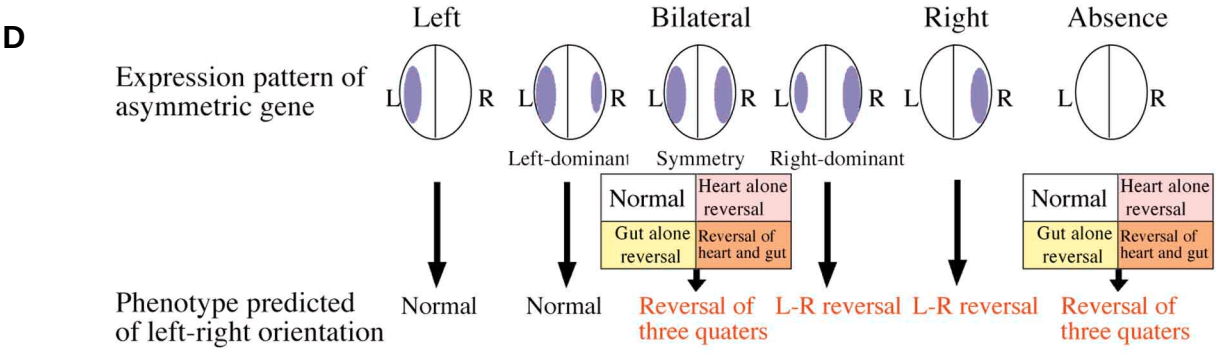


Fig. 5. Incidence of altered asymmetric gene expression and the predicted incidence of left-right organ reversal. *The blue, green, and red bars represent the expression of* Xnr-1, Xatv *and* Pitx2, *respectively. L, expression on the left only; L>R, bilateral expression that predominates on the left; L=R, symmetrical bilateral expression; L<R, bilateral expression that predominates on the right; R, expression on the right only; Absence, absence of expression.* **(A)** *Scoring of the embryos injected on the left at the neurula stage.* **(B)** *Scoring of the embryos injected on the right at the neurula stage. Right-side but not left-side injection induced gene expression in the right LPM and simultaneously repressed the normal left expression of these genes.* **(C)** *Scoring of the embryos injected on the right at the tailbud stage. Gene expression was induced in the right LPM but the normal left expression was not suppressed.* **(D)** *Calculation of the predicted incidence of left-right reversal on the basis of the altered expression patterns of the asymmetric genes caused by the injection of TGF-β5 on the right.*

Expected value of left-right reversal (%)

= (R)% + (L<R)% + (L=R)% x 3/4 + (Absence)% x 3/4

			L (%)	L>R (%)	L=R (%)	L<R (%)	R (%)	Absence (%)	***Expected value of L-R reversal (%)***
Injection into neurula embryos	*Xnr-1*	n=68	10	1	19	12	44	13	**80**
	Xatv	n=47	4	0	15	6	43	32	**84**
	Pitx2	n=47	4	2	4	11	70	9	**91**
Injection into tailbud embryos	*Xnr-1*	n=54	15	9	22	17	20	17	**66**
	Xatv	n=50	16	4	8	14	38	20	**73**
	Pitx2	n=57	26	32	25	11	7	0	**37**

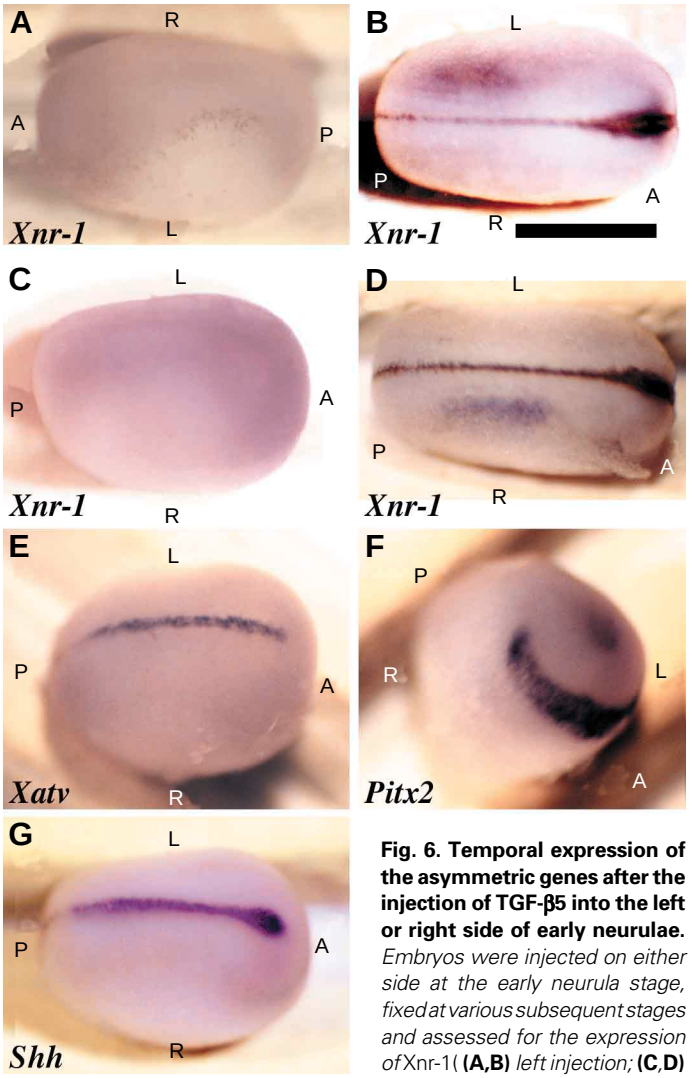


Fig. 6. Temporal expression of the asymmetric genes after the injection of TGF-β5 into the left or right side of early neurulae. *Embryos were injected on either side at the early neurula stage, fixed at various subsequent stages and assessed for the expression of* Xnr-1(**(A,B)** *left injection;* **(C,D)** *right injection),* Xatv (**(E)** *right injection), or* Pitx2 (**(F)**, *right injection). TGF-β5 injection could not induce the precocious expression of any of the genes in the mid-neurula embryos (stage 16; see A,C,E,F) and expression commenced at the normal time points (e.g.* Xnr-1 *was expressed at stage 19, see B,D).* **(G)** *Injection did not alter* Shh *expression along the midline or induce the ectopic expression of* Shh.

of the embryos showed left-handed *Xnr-1* expression and 50% (n=21/42) showed no expression at all (Fig. 9 A,F). *Pitx2* expression was affected similarly as *Xnr-1* expression (Fig. 9 C-D, F). TGF-β5 MO injected into the left dorsal blastomere resulted in 75% (n=30/40) of the embryos showing the absence of *Pitx2* expression in the left LPM.

TGF-β5 mRNA is expressed in the eye anlage at late tailbud stage (Kondaiah *et al.*, 2000; Fig. 8H). Probably in consistent with this, we found that TGF-β5 MO caused serious microphthalmia (reduced eye size phenotype) on the injection side. After injecting TGF-β5 MO into the dorsal blastomeres, eye size was greatly reduced and/or the differentiation of pigmented retina was inhibited in most of the injected embryos [right dorsal injection, 89% (n=70/79); left dorsal injection, 91% (n=88/97)](Fig. 8 A,B,I).

As a control experiment, we injected 2mM of anti-human globin MO or ribonuclease-free water into the dorsal blastomere of the 4-cell stage embryos, and assessed the left-right orientation. No significant left-right reversal was observed. Thus, the inhibition experiments suggest that endogenous TGF-β5 signaling may be involved in the left-right specification in *Xenopus* embryos.

Discussion

In the early chick embryo, many TGF-β-related ligands are known to be expressed asymmetrically with regard to the left-right axis (Levin *et al.*, 1995; Stern *et al.*, 1995; Levin *et al.*, 1997; Rodriguez-Esteban *et al.*, 1999; Monsoro-Burq and Le Douarin, 2000, 2001). However, in *Xenopus* embryos, only two left-handed TGF-β superfamily ligands, *Xnr-1* (Lowe *et al.*, 1996) and *Xatv* (Cheng *et al.*, 2000; Branford *et al.*, 2000), have been identified. Furthermore, right-handed genes in *Xenopus* embryos have not been documented. In the study reported here, we examined the role that TGF-β5 plays in the left-right specification of *Xenopus* embryos. We found that injection of TGF-β5 protein into the right side of the neurula markedly reverses the left-right orientation of the internal organs (Fig. 1B, 2A, Table 1). Furthermore, when we inhibited endogenous TGF-β5 signaling by administering antisense TGF-β5 MO on the left, this organ reversal was again observed (Fig. 8). These observations suggest that endogenous TGF-β5 signaling may be required for the normal left-right specification of *Xenopus* embryos.

Right-Sided TGF-β5 injection into the Neurulae drives Xnr-1 *Expression on the Right and blocks Normal Left-Handed Expression*

Injection of TGF-β proteins into the right side of neurulae induced the left-right reversal of the internal organs in up to 100% of the embryos (Figs. 1,2, Table 1).

Thus, the left-right specification that is underway in the neurula stage can be completely respecified by the TGF-β protein injection, indicating that the left-right differentiation at the neurula stage is not irreversible. However, when the right-side injection took place at the tailbud stage, the left-right orientation was inverted in only half of the embryos (Fig. 2, Table 1), indicating that the left-right specification that has taken place is becoming irreversible at the tailbud stage. These differences might correlate with the beginning of *Xnr-1* expression, which normally starts to be expressed in the late neurula at stage 19 (Lowe *et al.*, 1996). To date, six isoforms of the *Xenopus nodal-related genes* have been identified, all of which are involved in organizer formation (Jones *et al.*, 1995; Smith *et al.*, 1995; Lustig *et al.*, 1996; Joseph and Melton; 1997; Takahashi *et al.*, 2000). However, among them, only *Xnr-1* is known to be expressed on one side of the embryo from the late neurula stage onwards. Thus, it seems that TGF-β protein can completely reorient the left-right orientation when administered before the handed expression of *Xnr-1*, whereas, after the expression of *Xnr-1*, this ability is blocked by the original *Xnr-1* expression on the left side.

In fact, observations of gene expression pattern in the injected embryos support the above idea. For neurula embryos, right-sided injection of TGF-β5 induced right-dominant expression of *Xnr-1* (Fig. 5B). However, when the injection took place at the tailbud stage, bilateral expression was more commonly observed (Fig. 5C), indicating the original left expression had not been eliminated by the right-sided TGF-β5 injection.

We also found that normally left-handed expression of *Xatv* and *Pitx2* became right-handed after the right-sided TGF-β5 injection into the neurulae. It is likely that the *Xenopus nodal* gene *Xnr-1* induces the expression of *Xatv* (the *Xenopus lefty* gene) and *Pitx2*, because this cascade is conserved in all the vertebrates examined (Capdevila *et al.*, 2000). Supporting this is that *Xatv* and *Pitx2* were expressed later than *Xnr-1* in our temporal analysis of gene expression (Fig. 7). These observations suggest that the ectopically administered TGF-β protein first changed the laterality of *Xnr-1* expression, and this then drived the right-handed expression of *Xatv* and *Pitx2*. Thus, ectopically applied TGF may have only indirectly altered the laterality of *Xatv and Pitx2* expression.

Given that *Xnr-1* appears to be the primary determinant of left-right axis development, it is important to determine how endogenous TGF-β5 affects the *Xnr-1* expression *in vivo*. This is discussed in the following chapters.

A Putative Inhibitory Factor secreted from the Midline Tissues may regulate Xenopus *Neurula Left-Right Orientation*

An important observation is that injection of TGF-β5 at the neurula stage causes 100% left-right reversal of the internal organs and predominantly right-sided expression of *Xnr-1* (Figs. 2,3 and 5B). At the same time, surprisingly, original left-sided expression of this gene has been strongly suppressed by the right-side injection. This observation suggests that *Xnr-1* can potentially be expressed on either side but that in the normal embryo, the right-side expression of this gene is continuously suppressed for normal left-right development to take place.

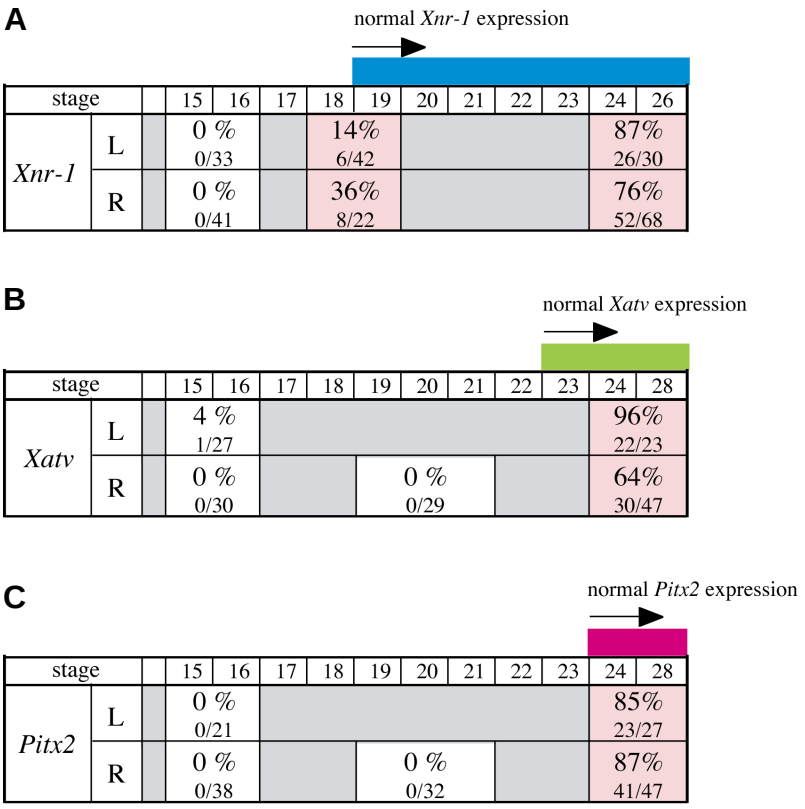


Fig. 7. Timing of expression of the asymmetric genes after TGF-β5 injection. *Early neurulae were injected with TGF-β5 and then fixed at various time points and their gene expression was scored. The scoring was focused on the expression in the LPM ipsilateral to the injection side. Significant (more than 10%) expression is colored pink; gray zone, not tested. Colored bars represent the time course of the expression of the handed genes in normal embryos (Lowe* et al.*, 1996; Ryan* et al.*, 1998; Cheng* et al.*, 2000).*

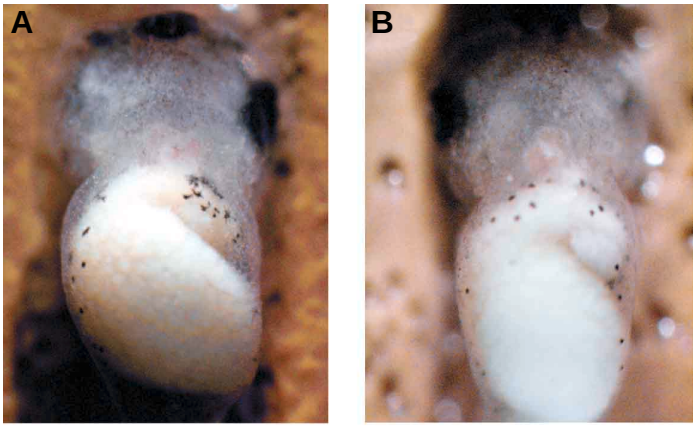
We propose that there is a suppressor of right-sided *Xnr-1* expression that may be secreted from the midline structure and diffuse into the LPMs. Supporting this idea, it has been reported that the explant of the right LPM that had dissected out from the midline structures at the mid-neurula stage showed unusual expression of *Xnr-1* (Lohr *et al.*, 1997; Levin and Mercola, 1998). These observations suggest that some inhibitory mechanism suppresses *Xnr-1* expression on the right side. Here we propose that right-side injection of TGF-β enhances the expression of this midline inhibitor so that it represses the endogenous left-handed *Xnr-1* expression. We suggest that, by the injection, this factor unusually diffuses into the left LPM to suppress the endogenous left-sided *Xnr-1* expression. The midline inhibitor probably also diffuses into the right LPM but the effect of the injected TGF-β5 protein may overcome the inhibitory effect of the midline factor. Conversely, in normal embryos, we suppose that left-side-specific event should overcome the inhibitory effect of the midline factor to initiate the asymmetric *Xnr-1* expression only on the left side.

We are currently searching for genes that are expressed in the midline and that are enhanced by TGF-β protein injection. *Xatv* (Cheng *et al.*, 2000; Branford *et al.*, 2000) and *Shh* (Ekker *et al.*, 1995) are known to be expressed in the midline tissues of neurula–tailbud *Xenopus* embryos, but we found that their expression is not changed by the injection (Fig. 6 E,G). Thus, these genes do not appear to be the candidates for the midline inhibitor.

Injection of TGF-β and Activin into Xenopus *Neurula Embryos has Different Effects*

Previously, we reported that injection of activin A protein into the left or right LPM of *Xenopus* early neurulae rapidly induced ectopic *Xnr-1* expression at the injection point by the mid-neurula stage (Toyoizumi *et al.*, 2000). On the other hand, we have found here that TGF-β5 injection into either side of neurulae does not induce such precocious expression of *Xnr-1* (Figs. 6, 7). In addition, activin was no longer able to alter the left-right orientation when injected at stage 25-26 in our previous report (Toyoizumi *et al.*, 2000), but TGF-β5 is still able to invert left-right orientation in up to 34% of the embryos when injected at the same stage (Table 1).

Why does the effect of activin injection differ from that of TGF-β5 injection? The signaling pathways for activin and TGF-β share common elements, but other components differ (Mathews and Vale, 1991; Mathews *et al.*, 1992; Massague, 1996, 1998). Activin ligand binds to the heterodimeric receptor composed of activin type I receptor (ALK-2 or ALK-4) and activin type II receptor (type IIA or type IIB receptor)(Massague, 1998; Massague *et al.*, 2000; Pangas and Woodruff, 2000), while TGF-β ligand binds to a combination of TGF-β type I receptor (ALK-1 or ALK-5) and TGF-β type II receptor (Lin *et al.*, 1992; Franzen *et al.*, 1993; Mahony and Gurdon, 1995; Mahony *et al.*, 1998; Massague *et al.*, 2000; Oh *et al.*, 2000; Dhanasekaran *et al.*, 2001). Though these ligands bind to different receptors, the downstream intracellular signal mediators are shared.



F

	D R L V	D R L V
TGF-β5 MO	3% 2/79	30% 29/97
Control MO (human globin MO)	0% 0/70	2% 1/64
RNase-free water	0% 0/25	0% 0/43

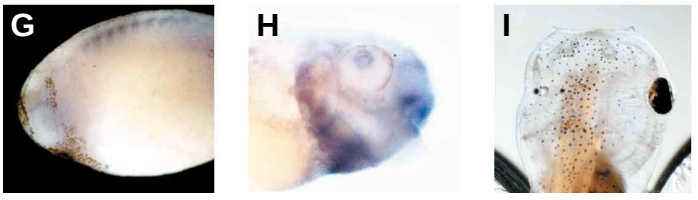


Fig. 8. Effect of injecting antisense morpholino oligonucleotide against TGF-β5. (A,B) *Right-side injection of* TGF-β5 *MO had no effect on left-right orientation of the internal organs (A, normal situs), whereas left-side injection reversed the orientation (B, the case of heart-alone reversal).* **(C,D,E)** *Injection of* TGF-β5 *MO into the dorsal blastomere inhibited the expression of* TGF-β5 *in somites in the early tailbud embryo. Bilateral expression of* TGF-β5 *(C, a control embryo) was down-regulated only on the injection side by injecting the MO. D, right side injection of the MO; the expression in somites was reduced or ceased in 60% of the embryos (n=18/30). E, left side injection; the paraxial expression was reduced or ceased in 57% of the embryos (n=20/35).* **(F)** *Shown are the ratios (%) of left-right reversal after injecting* Xenopus TGF-β5 *MO, human* globin *MO, and RNase-free water. The bottom numbers show the ratio of embryos with organ reversal/survived embryos. Only the case of left-side injection of* TGF-β5 *MO elicited significant left-right reversal.* **(G,H,I)** TGF-β5 *expression in the eye primordia and Morpholino knock-down of the pigmented retina differentiation. G, lateral view of a stage 23 embryo, which does not express* TGF-β5 *in the eye but expresses in somites. In later tailbud embryos,* TGF-β5 *is expressed in the inner margin of the retina primordia and the forming lens, nasal plate and branchial region (H). I, the larval "free lens" without retinal differentiation was frequently observed after the injection of the* TGF-β5 *MO.*

That is, both signaling is transduced by Smad2 and/or Smad3 (Wrana *et al.*, 1994; Wieser *et al.*, 1995; Lee *et al.*, 2001). After phosphorylation and coupling with Smad4, Smad2 and/or Smad3 move into the nucleus. Together with various downstream transcription factors, the Smad complexes then bind to the regulatory flanking regions of the targeted genes (Labbe *et al.*, 1998; Massague, 1998; Massague *et al.*, 2000).

In the case of activin signaling in *Xenopus* early embryos, one important partner of the R-Smads (Smad2/Smad3) in transcriptional regulation is XFAST (fork-head activin signal transducer: Chen *et al.*, 1996a, 1997; Watanabe and Whitman, 1999; Yeo *et al.*, 1999). XFAST-1 is a maternal factor, and the zygotic expression of *XFAST-1* mRNA is terminated at the late neurula stage (Howell *et al.*, 2002). Interestingly, FAST binding sites have been found in both the 5' upstream region and the first intron of *Xnr-1* (Watanabe and Whitman, 1999; Osada *et al.*, 2000). Recently, another member of XFAST, XFAST-3 was identified (Howell *et al.*, 2002). XFAST-3 acts cooperatively with XFAST-1 in *Xenopus* gastrulation. The transcription of XFAST-3 ceases by the end of gastrulation, while the transcription of XFAST-1 ceases before the tail bud stage (Howell *et al.*, 2002). Notably, the *Xenopus Pitx2* gene also has three binding sites for XFAST (Shiratori *et al.*, 2001). Based on these observations, we propose that the left-right reversal and the ectopic induction of *Xnr-1* by activin injection observed in our previous report may have been mediated by XFAST. The inability of the tailbud embryos to respond to activin injection may be due to the absence of both XFASTs at that stage.

Although the binding receptors of activin and TGF-β differ, the R-Smads and use of co-Smad (Smad4) for the intracellular signaling events are shared. That is, Smad2 and/or Smad3 are phosphorylated also in the case of TGF-β signaling (Chen *et al.*, 1996b; Heldin *et al.*, 1997; Nakao *et al.*, 1997). However, while FAST is known to be the indispensable signal transducer for activin signaling, the transcription factor(s) responsible for TGF-β-specific signaling in *Xenopus* is largely unknown. RT-PCR studies revealed that mRNAs of the two components of the heterodimeric TGF-β receptor are expressed during *Xenopus* neurula stage (Mahony and Gurdon, 1995; Dhanasekaran *et al.*, 2001). This suggests that some intracellular component of TGF-β signaling rather than the receptor molecules is absent in the embryo before the late neurula stage, which would explain why TGF-β5 can not induce *Xnr-1* expression before the late neurula stage (Figs. 6, 7).

Taken together, we propose that an unknown transcription factor other than XFAST may transduce the TGF-β signaling involved in *Xenopus* left-right specification. It is possible that some indispensable component of TGF-β-specific signaling is initially prepared only at the late neurula stage and that therefore the injected TGF-β can manifest its effect on *Xnr-1* expression only at this stage. Because the right-side injection of TGF-β5 induces the ectopic *Xnr-1* expression only at the normal timing when its endogenous expression starts, we expect that TGF-β5 is intimately

involved in the mechanism that drives the onset of asymmetric *Xnr-1* expression in the normal embryo.

Putative Mechanism by which TGF-β5 Signaling guides the Development of Left-Right Asymmetry

As shown in Fig. 8, inhibition of TGF-β5 signaling caused significant left-right reversal. Only the left-sided injection of TGF-β5 MO altered the laterality of the internal organs, hence we suppose that TGF-β5 signaling in the left side may be needed for the left-handed expression of *Xnr-1*. On the other hand, it is known that expression of *TGF-β5* mRNA is bilateral as far as investigated (Kondaiah *et al.*, 2000). It is therefore not clear how TGF-β5 can affect the expression of *Xnr-1* on the left side only. However, it may be that TGF-β5 acts similarly to other symmetrically-expressed molecules involved in left-right specification.

It is known that some members of TGF-β superfamily are expressed bilaterally, but regarded to be involved in the early determination of left-right asymmetry in vertebrates none the less. It has been reported that the Vg1 protein is involved in the initial formation of the left-right axis in the early *Xenopus* embryo, and today, many investigators believe that Vg1 is the most promising candidate as the left-right coordinator in *Xenopus* early development (Hyatt *et al.*, 1996; Hyatt and Yost, 1998; Ramsdell and Yost, 1999; Burdine and Schier, 2000). Vg1 is a maternal secretory protein and *Vg1* mRNA is expressed in the vegetal side of the embryos from the cleavage stage to the gastrula stage (Weeks and Melton, 1987; Tannahill and Melton, 1989). However, left-right preference in the localization of *Vg1* has not yet been detected. Therefore, it has been assumed that the processing of Vg1 protein is performed left-right asymmetrically, and that this mature Vg1 ligand then acts as the left-handed signal (Hyatt and Yost, 1998; Ramasdell and Yost, 1999). Supporting this are studies on the murine *growth differentiation factor-1* (*GDF-1*). This gene is highly homologous to *Vg1*, and *GDF-1* mRNA expression in the early mouse embryo is also symmetrical during the gastrula to the neurula stages (Rankin *et al.*, 2000). Physiological role of GDF-1 had been long unknown, but unexpectedly, in knockout mice of *GDF-1*, left-right axis of the internal organs was reported to be randomized (Rankin *et al.*, 2000). In addition, when *GDF-1* mRNA was injected into the single right blastomere of *Xenopus* cleavage-stage embryos, the left-right axis was inverted (Wall *et al.*, 2000).

In this report, we showed that TGF-β5 protein is able to induce left-right reversal in almost all of the embryos injected at the neurula stage. *TGF-β5* may act similarly to *Vg-1* and *GDF-1* in that while its mRNA expression is bilateral, active protein distribution is not. We know that *TGF-β5* is expressed symmetrically in the late organizer and then in the paraxial mesoderm by the time the injection is delivered (Kondaiah *et al.*, 2000). However, we found here that left-sided administration of *Xenopus TGF-β5* MO, which blocks endogenous TGF-β5 signaling, reverses the left-right orientation of the internal organs. Thus, it may be that the TGF-β5 preprotein is normally selectively spliced into an active form on the left side of *Xenopus* embryos, and this form then activates *Xnr-1* expression.

With regard to the notion that active TGF-β5 protein is only produced on the left side, Furin(SPC1)-like processing enzyme may be a candidate for this left-right asymmetrical TGF-β5 activation. In vertebrates, it has been reported that Furin is the mammalian prototype of a family of serine proteases that have catalytic domains resembling the bacterial protease Subtilisin (Roebroek *et al.*, 1986; Van de Ven *et al.*, 1993). Furin has been shown to process the precursor forms of the members of TGF-β superfamily including human TGF-β1 (Dubois *et al.*, 1995), *Drosophila* activin A (Roebroek *et al.*, 1993) and *Xenopus* BMP-4 (Cui *et al.*, 1998). Mouse *furin* mRNA is expressed in the cardiovascular and digestive systems at the late somite stage (Zheng *et al.*, 1994). In mice

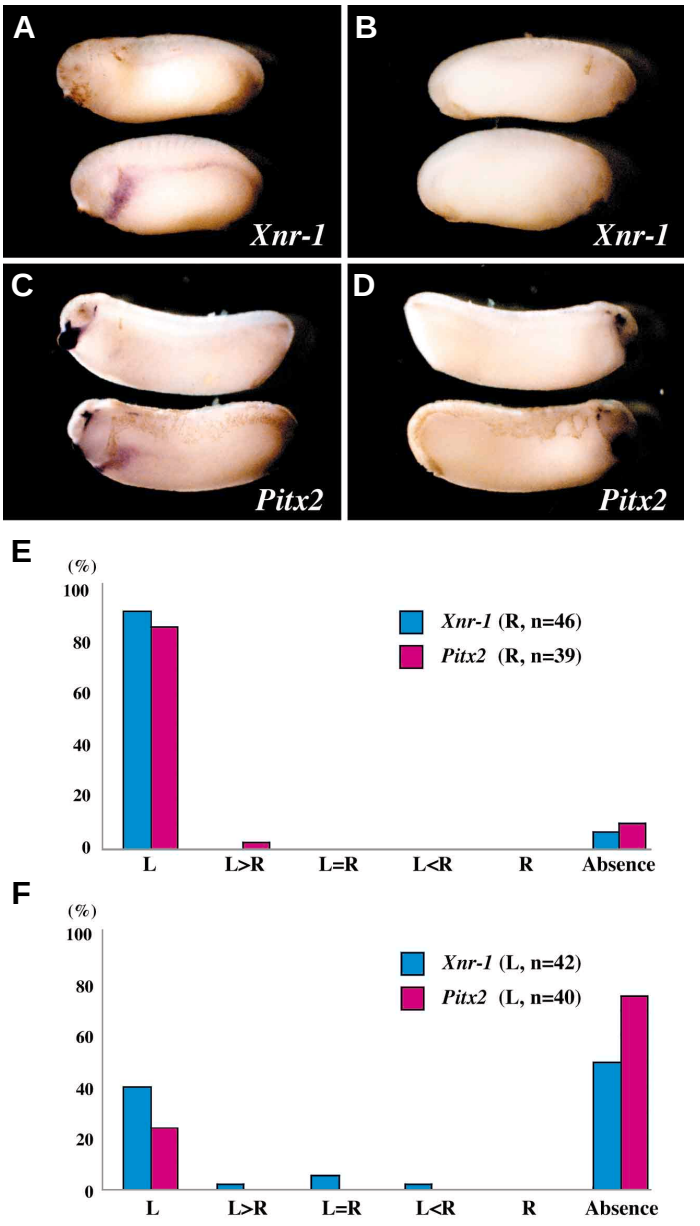


Fig. 9. Inhibition of handed gene expression by the injection of *TGF-β5* MO. **(A,B)** Xnr-1 *expression in embryos injected with* TGF-β5 *MO. The upper one was injected in the dorsal left blastomere, and ceases left-handed* Xnr-1 *expression (A, viewed from left side). In the lower one which was injected in the dorsal right blastomere, the expression of* Xnr-1 *in the left LPM is not affected.* **(C,D)** Pitx2 *expression in embryos injected with* TGF-β5 *MO. The upper one was injected in the dorsal left blastomere and, similarly to the case of* Xnr-1, *the embryo ceases left-handed* Pitx2 *expression (C, viewed from left side). The lower one was injected in the dorsal right blastomere, and left* Pitx2 *expression is not affected.* **(E,F)** *Embryos were scored for the expression of* Xnr-1 *(blue bar) and* Pitx2 *(red bar) after injecting* TGF-β5 *MO into the right (E) or left (F) blastomere. When injected on the left side,* TGF-β5 *MO downregulates the left-handed expression of* Xnr-1 *and* Pitx2.

lacking *furin*, inhibition of the expression of *Pitx2* resulted in the defect of embryonic turning (Constam and Robertson, 2000a). Furthermore, knockout mice of *PACE4* (*SPC4*), another member of the proprotein convertases that include *furin*, showed the *situs ambiguous* phenotype (disorder of left-right asymmetry; Constam and Robertson, 2000b). Until now, the processing enzyme(s) specific for TGF-β5 ligand has not been identified in *Xenopus* embryos. This putative processing enzyme may be a furin-like one and it may be selectively localized in the left tissues of *Xenopus* embryos, where it processes and activates the TGF-β5 ligand in the left side. The resulting TGF-β5 molecule can then act as the left-handed inducer of *Xnr-1* expression and thereby drive the asymmetrical signaling process leading to morphological left-right asymmetry. In the protein injection experiment conducted here, we used mature form of TGF-β5 protein and thus, without a help of the hypothetical processing enzyme localized on the left side, ectopic induction of *Xnr-1* and sequential activation of downstream *Xatv* and *Pitx2* was performed on the right side.

Materials and Methods

Xenopus *Eggs and Microinjection*

Fertilized *Xenopus laevis* eggs were obtained from a couple of adult male and female injected with gonadotropic hormone. The jelly layer was removed by treatment with 2.5% thioglycolic acid (pH 8.6) at the cleavage stage and the embryos were cultured in sterile 10% Steinberg's solution at 15-18ºC before microinjection. Their developmental stages were identified according to the normal developmental table of Nieuwkoop and Faber (1967).

For microinjection, the embryos were de-chorionized and transferred into a Terasaki plastic plate (Sumitomo Bakelite Co.) filled with the saline. For the injection of stage 25-34 tailbud embryos, the embryos were first transferred for a few minutes into an organ culture dish (Falcon 3037, Becton Dickinson Co.) filled with the saline containing 0.01% anesthetic drug (MS-222). Neurulae and tailbud embryos were injected hypodermically in the center of their left or right flanks with 5 nl or 25 nl of various protein solutions using an electric microinjector (Drummond Co. 'Nanoject'). To verify the injection side at a later stage, the protein solution was mixed with 10% Nile Blue vital dye solution (Wako Co.) at a ratio of 10:1 in all cases.

The following proteins were injected mainly at concentrations of 2 or 10 μg/ml: TGF-β1 (porcine TGF-β1, R & D systems Co.), TGF-β2 (recombinant human TGF-β2, Wako Co.; porcine TGF-β2, R & D systems Co.), TGF-β3 (recombinant human TGF-β3, R & D systems Co.) and TGF-β5 (recombinant *Xenopus laevis* TGF-β5, R & D systems Co.).

Various growth factors other than TGF-β family were also injected into one side of early-mid neurulae. The following proteins were injected: fibroblast growth factor(FGF)-8 (recombinant mouse FGF-8, R & D systems Co; recombinant human FGF-8, Pepro Tech Ins.), FGF-10 (recombinant human FGF-10, R & D systems Co.; recombinant human FGF-10, Pepro Tech Ins.), insulin-like growth factor(IGF)-I (recombinant human IGF-I, Pepro Tech Inc.), IGF-II (recombinant human IGF-II, Pepro Tech Inc.), platelet derived growth factor(PDGF) (recombinant human PDGF-AA, R & D systems Co.), vascular endothelial growth factor(VEGF) (recombinant rat VEGF, R & D systems Co.), growth differentiation factor(GDF)-6 (recombinant mouse GDF-6, R & D systems Co.) or bone morphogenetic protein(BMP)-3 (recombinant human BMP-3, R & D systems Co.). All were injected at 500 pg or more except FGF-8, which was injected at 250 pg or 500 pg.

For the control experiments, 5 nl of 1% BSA solution (bovine serum albumin fraction V, Sigma Co.) was injected. After microinjection, the embryos were transferred into individual wells of 24-well test plates containing the saline with 0.1% BSA (Iwaki Glass Co.). They were then cultured at 18-26ºC until they reached stage 41-42. At this point, left-right orientation of the heart and visceral organs were scored according to the criteria as previously described (Toyoizumi *et al*., 1997, 2000). To more precisely judge the gut asymmetry, the laterality of the ventral pancreas was also checked. The embryos were also scored for the morphogenetic pattering along the antero-posterior and dorso-ventral axes using the dorso-anterior index (DAI; Kao and Elinson, 1988).

To inhibit endogenous TGF-β5-specific signaling, 10 nl of 1mM antisense MO targeted to *Xenopus TGF-β5* (5'-CCA GCA GCA TCC ACA GAA CCT CCA T-3'; designed to hybridize to the CDS 504...528 of GenBank No. J05180 [CDS 504..1652]; Gene Tools, LLC) was injected into one of the dorsal blastomeres of 4-cell stage embryos. In *Xenopus*, *TGF-β5* and *TGF-β2* (X51817; Rebbert *et al.,* 1990) have been cloned and genes of other TGF-βs have not yet been cloned. The Morpholino probe against TGF-β5 used in this report has very low complementarity to *TGF-β2* mRNA with 9-mispairing (36% mismatch) even at the fittest sequence. As a control oligonucleotide, 5nl of 2mM antisense MO against human globin gene (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3') was injected. Immediately after the injection, the embryos were placed in the saline containing 4% Ficoll (ICN Biomedicals Inc.), cultured overnight, and then transferred into the saline lacking Ficoll and cultured further at 18-26ºC until they had reached stage 41-42.

Calculation of the Incidence of Left-Right Reversal in the Injected Embryos

Both the injected embryos and the uninjected sibling embryos were observed at stage 41-42 to judge the left-right orientation of the heart and visceral organs. They were classified into 4 phenotypes, namely, normal, reversed heart and gut, reversed heart only, and reversed gut only. The latter three phenotypes were all counted as "left-right reversal of the internal organs" and the incidence of left-right reversal was calculated using the following equation:

> [incidence of left-right reversal (%)] = [(number of embryos inverted in both heart and gut)+(number of embryos inverted in heart only)+(number of embryos inverted in gut only)] ÷ [number of surviving embryos] x 100

Whole-Mount In Situ *Hybridization*

cDNAs of *Xenopus nodal related-1* (*Xnr-1*) and *Xenopus Sonic hedgehog* (*Shh*) were gifted from Dr. Randall T. Moon (University of Washington). cDNA of *Xenopus lefty-related factor antivin* (*Xatv*) was gifted from Dr. Christopher V. E. Wright (Vanderbilt University). cDNA of *Xenopus Pitx2c* (*Pitx2*) was gifted from Dr. Juan Carlos Izpisúa Belmonte (The Salk Institute). cDNAs of *Xenopus Mad2* (*Smad2*) and *Xenopus TGF-β5* (*TGF-β5*) were gifted from Dr. Douglas A. Melton (Harvard University).

The embryos for whole-mount *in situ* hybridization were fixed with MEMFA at room temperature for one hour, washed with TBST, dehydrated with methanol, and stored in methanol at -20ºC until the staining. The expression patterns of *Xnr-1*, *Xatv*, *Pitx2*, *Smad2*, *TGF-β5* and *Shh* genes were examined by whole-mount *in situ* hybridization according to Harland's methods (1991) with slight modification. Antisense RNA probes and control sense probes labeled with digoxigenin were synthesized. Stained embryos were washed with PBS- containing 0.1% Tween 20, photographed, and stored in 50% glycerol at 4ºC.

The expression patterns of these genes in the injected or normal embryos were classified into six classes, namely, expression on the left side only, expression on the right side only, bilateral LPM expression but with the left dominating (left > right), symmetrical bilateral expression (left = right), bilateral expression but with the right dominating (left < right), and no expression in either LPM (Fig. 5D).

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