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Original Article

Xenopus nodal related-1 is indispensable only for left-right axis determination

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ABSTRACT In *Xenopus*, multiple *nodal*-related genes are expressed in the organizer region. Among them, only *Xenopus nodal related-1* (*Xnr-1*) is expressed unilaterally in the left lateral plate mesoderm (LPM) at late neurula-early tailbud stage. To elucidate the essential role of *Xnr-1* for left-right specification, loss of function experiments using antisense morpholino oligonucleotides (MOs) targeting three different regions of *Xnr-1* were performed. Left-side injection of *Xnr-1* MO suppresses the left-side specific genes such as *Xnr-1*, *Xenopus antivin* (*lefty*) and *Xenopus pitx2* and randomizes cardiac and visceral left-right orientation. In contrast, paraxial bilateral expression of *Xnr-1* along the posterior notochord is not affected by the *Xnr-1* MO. In embryos injected with the *Xnr-1* MO, morphology of dorsal axial structures is normal and dorsal expression of *sonic hedgehog* and *TGF-β5* is not changed. Right-side injection of Nodal protein, or polyethyleneimine-based gene transfer of *Xnr-1* mRNA in the right LPM induces *Xnr-1* and *pitx2* in the same side and fully (more than 90%) reverses situs of the internal organs. Left-side injection of Nodal protein restores normal left-right orientation in the embryos that were injected with *Xnr-1* MO into the left blastomere and would cause randomization of the left-right axis without the Nodal injection. Taken together, unilateral expression of *Xnr-1* in the left LPM directs the orientation of the left-right axis by driving the left-specific gene cascade. Knockdown of Xnr-1 function by the MOs suggests that Xnr-1 is indispensable only for the left-right orientation and dispensable for other embryonic axes probably owing to the redundancy in the function of multiple *Xnrs*.

KEY WORDS: *nodal, morpholino, Xenopus, neurula, left-right asymmetry*

Introduction

The *nodal* gene encodes a ligand of TGF-β superfamily, a family of signaling peptides. In general, Nodal protein acts as a homodimer of approximately 40 kDa and mediates TGF-β-related intracellular signaling (Schier and Shen, 2000; Shier, 2003). Historically, *nodal* was first cloned from the cDNA library of a mouse ES cell line and was reported to be expressed in the mouse organizer region (Zhou *et al*., 1993; Conlon *et al*., 1994). In *nodal*-deficient mouse embryos, the primitive streak and mesoderm layer are not formed and these embryos die at 7.5 dpc. Thus, *nodal* proved to be crucial for mesoderm induction in the mouse embryo. In human, mouse, rabbit and rat embryos, only one *nodal* gene has been identified. Without exception, all the known mammalian *nodal* genes are expressed in the organizer region of the embryos (Fujinaga *et al*., 2000; Fischer *et al*., 2002).

nodal-related genes have also been identified in other vertebrate species. In 1995, *chick nodal related-1* (*cnr-1*) was reported to be expressed left-right asymmetrically in both the organizer region and the lateral plate mesoderm (LPM)(Levin *et al*., 1995, 1997). In these regions, *cnr-1* is expressed transiently, predominantly in the left side at the early somite stage. Subsequently, both mouse *nodal* and *Xenopus nodal related-1* were found to be expressed in the left LPM and not in the right LPM (Collignon *et al*., 1996; Lowe *et al*., 1996). Mouse *nodal* is expressed bilaterally on the peripheral margin of the node at first and then predominantly expressed on the left peripheral region of the node (Collignon *et al*., 1996; Lowe *et al*., 1996), while *Xnr-1* expression adjacent to the late organizer region is completely bilateral continually during late neurula–early tail bud stage (Lowe *et al*., 1996; Lustig *et al*., 1996; this report). In zebrafish, three *nodal-related* genes known as *squint, cyclops* and *southpaw* have been reported. Zebrafish *cyclops* and *southpaw* are expressed in the left LPM (Rebagliati *et al*., 1998a; Rebagliati *et al*., 1998b; Long *et al*.,

Abbreviations used in this paper: LPM, lateral plate mesoderm; MO, morpholino oligonucleotide; TGF, transforming growth factor; Xnr, Xenopus nodal-related.

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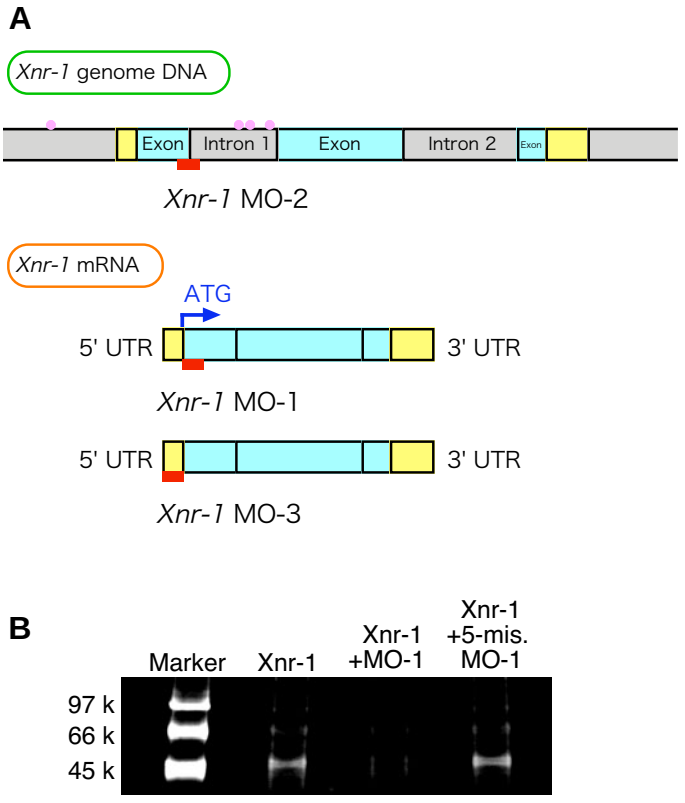


Fig. 1. Morpholino oligonucleotides (MOs) designed for knockdown of Xnr-1 and inhibition of Xnr-1 protein synthesis by the MO. (A) *Structure of the* Xnr-1 *gene and target regions of the MOs. Exons, introns and UTRs are colored in blue, gray and yellow, respectively.* Xnr-1 *gene has three exons and two introns and the transcript is 1500bp. FAST (forkhead activin signal transducer)-binding sites are marked by red circles (Osada* et al.*, 2000).* Xnr-1 *MO-1 is hybridized to the translation start codon region.* Xnr-1 *MO-2 is hybridized to the splicing region at the interface of the first exon and first intron.* Xnr-1 *MO-3 is hybridized to the 5'-UTR region.* Xnr-1 *MO-2 and MO-3 are labeled with FITC at their 3'-end.* **(B)** *Inhibition of* in vitro *translation of fluorescently labeled Xnr-1 protein by* Xnr-1 *MO-1. Fluorescent Lysine was added to the translation reaction of* Xnr-1*. The molecular weight of the fluorescent product is approximately 50kDa. Lane 1, no supplement of* Xnr-1 *MO-1; Lane 2, supplement of* Xnr-1 *MO-1; Lane 3, supplement of* Xnr-1 *5-mismatch MO-1 (control MO).*

2003). *cyclops* is expressed transiently in the presumptive region of the left dorsal epithalamus of the diencephalon (Rebagliati *et al.*, 1998a; Rebagliati *et al.*, 1998b; Liang *et al.*, 2000). *southpaw* induces the expression of *cyclops* in the left LPM and *southpaw* is necessary for *cyclops* expression in the left diencephalon (Long *et al.*, 2003; Ahmad *et al.*, 2004). Thus, *southpaw* is currently the earliest molecular marker of left-right asymmetry in zebrafish and functional in both visceral and diencephalic asymmetry.

Interestingly, as many as six *nodal-related* genes have been identified in *Xenopus laevis* embryos (Jones *et al.*, 1995; Smith *et al.*, 1995; Joseph and Melton, 1997; Takahashi *et al.*, 2000). All the *Xnrs* except *Xnr-5* are expressed in the dorsal lip of the gastrulating embryo. Among these *Xnrs*, only *Xnr-1* is known to be expressed unilaterally in the left LPM and the expression patterns of other *Xnrs* do not show left-right asymmetry. Transcripts of *Xnr-1* is first detected at the late blastula stage over the entire vegetal region, then its signal is restricted to the dorsal marginal zone at the early gastrula stage (Jones *et al.*, 1995). Its expression is not detected in early-mid neurula embryos, but expression is again found at the late neurula stage (Jones *et al.*, 1995). Bilateral posterior paraxial expression appears and, soon afterwards, left-specific LPM expression is recognized at the late neurula stage (Lowe *et al.*, 1996; Lustig *et al.*, 1996). Unilateral left LPM expression propagates from the dorsal posterior end of the LPM to the entire region of the LPM; *Xnr-1* expression is then restricted to the ventral anterior tip of the LPM juxtaposed to the left heart primordium (Lowe *et al.*, 1996). This expression pattern in the left LPM resembles that of *southpaw* in zebrafish embryos (Long *et al.*, 2003). In contrast to mammalian and chick *nodal*, *Xnr-1* expression in both the dorsal organizer region and later posterior paraxial region, is symmetrical.

Early studies reported that injecting high doses of *Xnr-1* mRNA, or other *Xnr* mRNAs, into *Xenopus* cleavage-stage embryos resulted in formation of a secondary axis having dorsal axial structures (Jones *et al.*, 1995; Takahashi *et al.*, 2000; Yamamoto *et al.*, 2001; Onuma *et al.*, 2002). These results are consistent with animal cap assay experiments, which demonstrated that *Xnrs* have an inducing activity for dorsal mesodermal tissues (Jones *et al.*, 1995; Joseph and Melton, 1997; Reissmann *et al.*, 2001). Injecting *Xnr-1* expression plasmid having EF1α promoter (pXEX; driving expression of *Xnr-1* RNA from late blastula) or cytoskeletal actin promoter (pCSKA; driving expression of *Xnr-1* RNA from early gastrula) into the right hemisphere of the cleavage stage embryos reversed the laterality of both the heart and gut (Sampath *et al.*, 1997). Injection of *Vg1*, *activin* or *Derrière* mRNA (TGF-β superfamily ligands) into the right hemisphere also fully inverted the internal organs (Hyatt *et al.*, 1996; Hyatt and Yost, 1998; Hanafusa *et al.*, 2000). Bilateral injection of an *Xnr-1* expressing vector into 8-cell stage *Xenopus* embryos caused bilateral LPM expression of *pitx2*, a candidate for a downstream target gene of left-sided *Xnr-1* expression (Ryan *et al.*, 1998; Campione *et al.*, 1999). However, redundancy in the function of multiple *nodal-related* genes in both zebrafish and *Xenopus* and complex ligand-receptor affinities in TGF-β related signaling make it difficult to analyze precise *Xnr-1* function only via gain-of-function studies. We are afraid that a ligand of TGF-β superfamily mimics the endogenous role of another ligand in overexpression studies. Therefore, we examined the effect of antisense morpholino oligonucleotides (MOs) specific for *Xnr-1* on the laterality of the left-specific gene expression and the placement of the internal organs.

First, we show evidence that *Xnr-1* expression in the left LPM is essential for the orientation of left-right asymmetry of the internal organs. The antisense *Xnr-1* MO suppressed the left-handed genes *Xnr-1*, *Xenopus antivin* (*lefty*) and *Xenopus pitx2* and randomized left-right orientation, but isomeric (left-right symmetric) development of the internal organs was rarely observed. The expression of two dorsal axial markers (*sonic hedgehog* and *TGF-β5*) was not affected by the MO. Thus, we hypothesize that *Xnr-1* in the left LPM controls the orientation of the left-right axis rather than simply conferring leftness.

Secondly, we changed the left-right balance of Nodal signaling between the two LPMs through administration of Nodal protein or gene transfer of *Xnr-1* mRNA using polyethyleneimine into the right flank of *Xenopus* neurula embryos. Activation of Nodal signaling in the right LPM affected the laterality of *Xnr-1* and *pitx2* and caused

a high level (more than 90%) of left-right reversal of the heart and gut. Furthermore, left-side injection of Nodal protein at neurula stage rescued the phenotype of *situs inversus* (randomization of left-right orientation) caused by the *Xnr-1* MO.

Altogether, using two complementary approaches, we proved the indispensable role of unilateral *Xnr-1* signaling in the left LPM in the specification of left-right orientation. Importantly, knockdown of Xnr-1 signaling by each of the three MOs did not affect the outer shape and dorsal axial structures of *Xenopus* larvae, strongly suggesting that Xnr-1 is not indispensable for early development except for the left-right axis determination.

Results

Antisense morpholino oligonucleotides for Xnr-1 *randomize the organ situs*

Injection of three MOs targeted to *Xnr-1* (*Xnr-1* MO-1, -2 and -3) was performed at the 4-cell stage (Figs. 1, 2). These MOs recognize the three different regions of *Xnr-1* gene (see Materials and Methods). *In vitro* translation experiment using fluorescent Lysine revealed that *Xnr-1* MO-1 inhibited the protein synthesis of *Xnr-1* (Fig. 1B). After injecting *Xnr-1* MO-2 labeled with FITC into the left ventral blastomere of a 4-cell stage embryo, only left side of the neurula embryo including the left flank was illuminated (Fig. 2A-D), which shows the distribution of the MO-2. When the left ventral blastomere was injected with the *Xnr-1* MO-1, 68% of the embryos showed reversal of left-right asymmetry of the internal organs (n=180, Figs. 2E, 3A and Table 1). Injecting the left dorsal blastomere caused 67% left-right reversal (n=86, Fig. 3A and Table 1). By contrast, injection into the right dorsal and/or right ventral blastomere caused less than 9% left-right reversal (n=220; Figs. 2E, 3A and Table 1). In the case of left-side injection of *Xnr-1* MO-2 or *Xnr-1* MO-3, left-right reversal at similar frequency was also observed (Figs. 2F, G and 3E, F). Statistical analysis using 2x2 contingency table test demonstrated that the left-right difference in the effect of each *Xnr-1* MO on organ situs is significant at 0.1% significance level (χ=179.5, 144.9, 95.8 respectively). Left-right symmetric morphology of the heart or gut was minimally observed after the *Xnr-1* MO injection at the 4-cell stage (Fig. 3A, E, F). Left-side injection of *Xnr-1* MO-1 frequently caused three

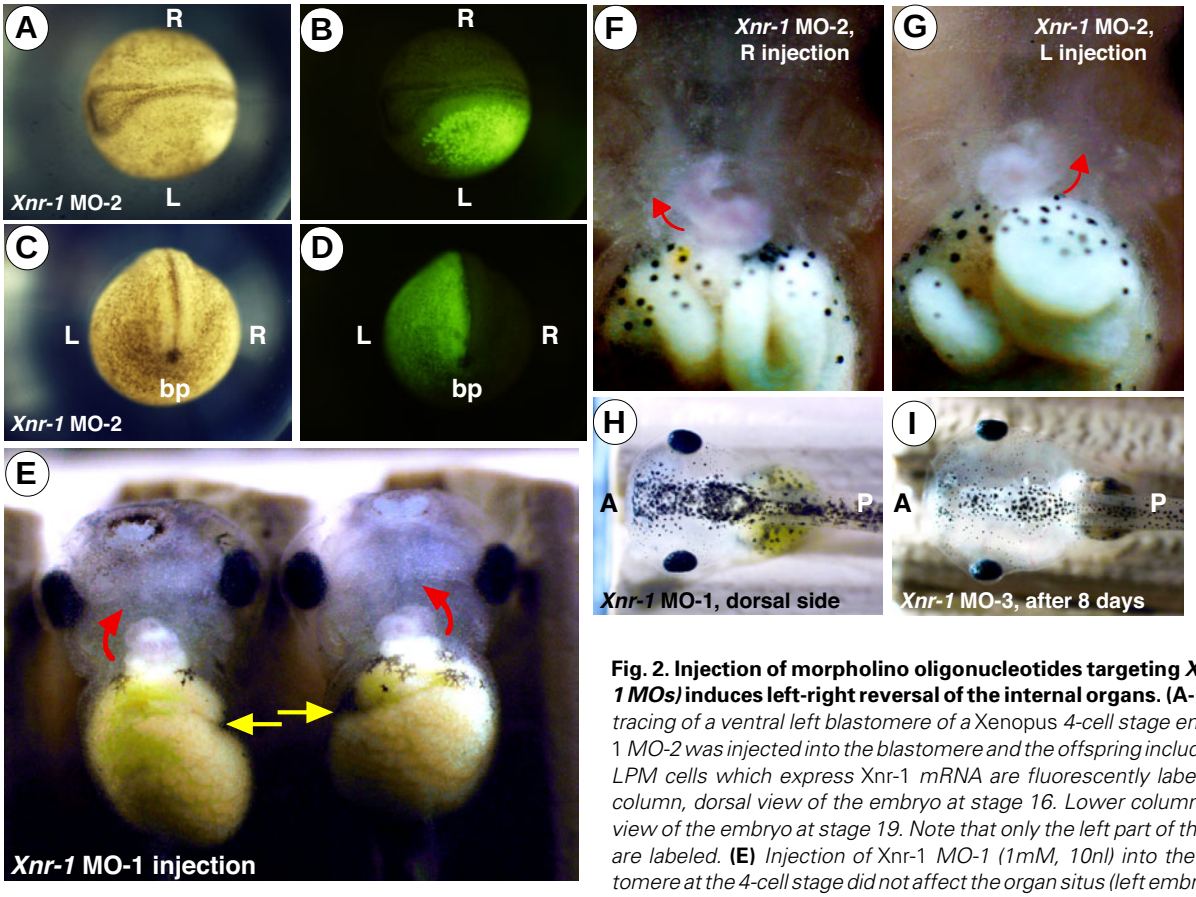


Fig. 2. Injection of morpholino oligonucleotides targeting *Xnr-1 (Xnr-1 MOs)* induces left-right reversal of the internal organs. (A-D) *Lineage tracing of a ventral left blastomere of a* Xenopus *4-cell stage embryo.* Xnr-1 *MO-2 was injected into the blastomere and the offspring including the left LPM cells which express* Xnr-1 *mRNA are fluorescently labeled. Upper column, dorsal view of the embryo at stage 16. Lower column, posterior view of the embryo at stage 19. Note that only the left part of the embryos are labeled.* **(E)** *Injection of* Xnr-1 *MO-1 (1mM, 10nl) into the right blastomere at the 4-cell stage did not affect the organ situs (left embryo at stage 42), while injection into the left blastomere at the same dose caused the left-right reversal in both the heart and gut (right embryo). Note that the morphology of the internal organs is normal except for the left-right orientation. Red curved arrows show the direction of blood flow. Yellow arrows indicate the curvature of the gut.* **(F,G)** *Stage 44/45 embryos after injecting with* Xnr-1 *MO-2 (1mM, 10nl) into the ventral right (F) or ventral left (G) blastomere at the 4-cell stage. The embryo in (F) shows normal situs, while the embryo in G shows the left-right reversal of the heart.* **(H)** *Dorsal view of a stage 42 embryo after injecting with 5nl of 1mM* Xnr-1 *MO-1 into each one of the two dorsal blastomeres at the 4-cell stage. Morphology of the outer figure is normal, suggesting that the redundancy in the function of multiple Xnrs has rescued the embryo from abnormal morphogenesis.* **(I)** *Dorsal view of a eight-day tadpole after injecting with* Xnr-1 *MO-3 into the dorsal left blastomere at the 4-cell stage. Morphology of the dorsal structures is symmetric and normal.*

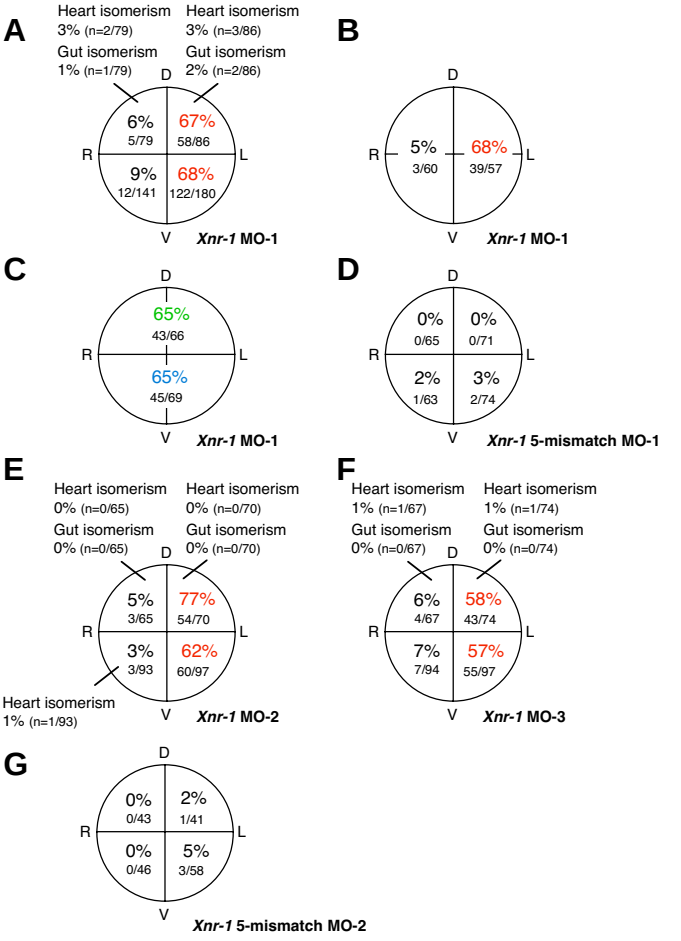


Fig. 3. Occurrence of left-right reversal after the *Xnr-1* MO-1 (A, B, C), 5-bp mismatch MO-1 (D), MO-2 (E), MO-3 (F) and 5-bp mismatch MO-2 (G) injections at the 4-cell stage. *In all the illustrations, the incidence of left-right reversal is shown as a percentage of [number of the embryos showing heart and/or gut left-right reversal]/[number of the embryos examined for organ situs].* **(A)** *The incidence of left-right reversal of the organ situs in the embryos injected with* Xnr-1 *MO-1 at the 4-cell stage. After the injection into either the dorsal-left or ventral-left blastomere, Xnr-1-deficient embryos showed more than 67% left-right reversal, while the right-side injections at the same dose caused only a low incidence of the reversal. Left-right symmetric phenotypes of the internal organs are rarely observed.* **(B,C)** *The incidence of left-right reversal in the case of* Xnr-1 *MO-1 injection into two left or right blastomeres (B) and two dorsal or ventral blastomeres (C). Five nanoliter of 1mM* Xnr-1 *MO was injected into each of two blastomeres and the injected embryos showed* situs inversus *when the Xnr-1 activity was downregulated in the left half of the embryos. The incidence of left-right reversal after injecting into two dorsal or ventral blastomeres was almost the same with the case of the injection into a single left blastomere, suggesting that right-side injection of* Xnr-1 *MO did not enhance or reduce the effect of the left-side MO injection.* **(D)** *Five-bp mismatch* Xnr-1 *MO-1 (control MO) caused minimal effects on the organ situs, showing that the effect of* Xnr-1 *MO-1 is specific to the translation of* Xnr-1 *mRNA.* **(E,F)** *Both* Xnr-1 *MO-2 and MO-3 are effective on the organ situs only when they are injected into the left blastomeres. Incidence of the left-right reversal after the left-side injections ranges from 57 to 77%, suggesting that randomization in the orientation of left-right axis has occurred.* **(G)** *Five-bp mismatch* Xnr-1 *MO-2 did not induce significant left-right reversal, demonstrating that* Xnr-1 *MO-2 is specifically targeted for* Xnr-1 *mRNA.*

types of *situs inversus*; that is, reversal of both heart and gut, reversal of heart-alone with normal gut situs and reversal of gut-alone with normal heart situs (Table 1). These observations suggest that randomization of heart situs and gut situs occurred independently after *Xnr-1* MO injection. The pattern of the organ reversal after injecting with *Xnr-1* MO-2 or *Xnr-1* MO-3 was similar to that caused by *Xnr-1* MO-1.

One-sided injection of *Xnr-1* MOs did not affect the outer shape of the embryo; thus, teratological effects such as the inhibition of gastrulation did not occur in Xnr-1-deficient embryos. Interestingly, *Xnr-1* MO-1 injection into two dorsal blastomeres of 4-cell stage embryos allowed the embryos to undergo normal morphogenesis with the exception of the left-right orientation of the internal organs (Fig. 2H and Table 1). Probably, redundancy in the function of multiple *nodal-related* ligands in *Xenopus* embryos covered the injected embryos from the deficiency of Xnr-1 signaling during gastrulation. For all the three *Xnr-1* MOs, the morphology of the heart and visceral organs in MO-injected larvae was normal except for the handedness of these organs, suggesting that *Xnr-1* MO affected mainly the laterality of the left-right signaling (Fig. 2E, F, G). In favor of this idea, survival ratio of the Xnr-1 MO-injected larvae was nearly equal to the untreated siblings. When injected with *Xnr-1* MO-2, 132 out of 162 injected larvae (82%) had survived for 10 days, while survival ratio of the siblings was 98% (n=94/96). For *Xnr-1* MO-3, 153 out of 163 injected larvae (94%) had

TABLE 1

THE SURVIVAL RATIO AND THE INCIDENCE OF LEFT-RIGHT REVERSAL OF THE INTERNAL ORGANS AFTER INJECTING AN ANTISENSE MORPHOLINO OLIGONUCLEOTIDE TARGETING *XNR-1*

Stage	Survival of injected embryos	Total incidence of L-R reversal	L-R reversal of both heart and gut	L-R reversal of heart-alone	L-R reversal of gut-alone (%)
	59 79/133	6 5/79	0 0/79	6 5/79	0 0/79
	80 86/108	67 58/86	20 17/86	19 16/86	29 25/86
	95 141/148	9 12/141	3 4/141	5 7/141	1 1/141
	85 180/212	68 122/180	17 31/180	23 41/180	28 50/180
	100 60/60	5 3/60	2 1/60	3 2/60	0 0/60
	95 57/60	68 39/57	25 14/57	12 7/57	32 18/57
	93 66/71	65 43/66	20 13/66	24 16/66	21 14/66
	95 69/73	65 45/69	19 13/69	25 17/69	22 15/69

When the left blastomere was injected with *Xnr-1* MO-1, both the heart and gut caused frequent left-right reversal.

TABLE 2

SUMMARY OF THE QUANTITATIVE ASSESSMENT FOR THE INHIBITION OF EXPRESSION OF NORMALLY LEFT-HANDED GENES AFTER *XNR-1* MO-1 OR CONTROL *XNR-1* 5-MISMATCH MO-1 INJECTION

		***Xnr-1* MO-1 injection**		***Xnr-1* 5-mismatch MO-1 injection**	
					
Xnr-1	Left	94% (n=34/36)	39% (n=13/33)	95% (n=37/39)	100% (n=36/36)
	Absence	6% (n=2/36)	61% (n=20/33)	5% (n=2/39)	0% (n=0/36)
Xatv	Left	90% (n=26/29)	22% (n=7/32)	-	-
	Absence	10% (n=3/29)	78% (n=25/32)	-	-
Pitx2	Left	93% (n=25/27)	0% (n=0/32)	-	-
	Absence	7% (n=2/27)	100% (n=32/32)	-	-

Expression of *Xnr-1*, *Xenopus antivin* and *Xenopus pitx2* was greatly reduced only when the embryos were injected with the *Xnr-1* MO into the left blastomere, suggesting that without left-side expression of *Xnr-1*, left-right orientation of the internal organs is randomized. *Xnr-1* 5-mismatch MO-1 did not affect the expression of *Xnr-1* in the left LPM.

survived for 10 days, while survival ratio of the siblings was 98% (n=94/96).

Observations of the cross sections of *Xnr-1* MO-injected larvae showed that the dorsal axial structures appeared to be normal and the ventral internal tissues also appeared to be normal except for the left-right orientation (Fig. 4D).

Xnr-1 *MO inhibits left-handed gene expression*

In support of the morphological observations, injection of *Xnr-1* MO-1 did not change the organizer/axial expression of *sonic hedgehog* and the paraxial expression of *TGF-β5* (Fig. 4A, B, C; Ekker *et al.,* 1995; Kondaiah *et al.,* 2000; Mogi *et al.,* 2003). Next, using the *Xnr-1* MO, we examined the effect of *Xnr-1* MO on left-handed gene expression (Fig. 5 and Table 2). *Xnr-1* expression in the left lateral plate mesoderm was greatly reduced after the left-side *Xnr-1* MO-1 injection, while the right-side injection of *Xnr-1* MO-1 did not change the laterality of *Xnr-1* expression (Fig. 5A, B and Table 2). Prior to the unilateral *Xnr-1* expression in the left LPM, bilateral paraxial expression of *Xnr-1* is observed at the posterior dorsal site neighboring the chordaneural hinge (Lowe *et al.*, 1996). This symmetric *Xnr-1* expression was not affected by *Xnr-1* MO, suggesting that *Xnr-1* expression in this area is independent of Xnr-1 signaling in the left LPM and *not* maintained by the *Xnr-1* autoregulatory loop (Fig. 5I and Table 3). *Xnr-1* MO-1 also reduced the left-sided expression of both *Xenopus antivin* and *pitx2* (Fig. 5E-H and Table 2). These two genes have been regarded as downstream targets of *Xnr-1* and this result supports this model. Left-side injection of *Xnr-1* MO-1 did not suppress the axial *antivin* expression (Table 4), suggesting that *Xnr-1* in the left LPM does not affect the axial *antivin* expression.

In order to demonstrate that *Xnr-1* MO-1 specifically inhibits the translation of *Xnr-1* mRNA, a control experiment using a mismatched complementary strand was performed. A *Xnr-1* 5-mismatch MO-1, which has sequence mismatches at five residues compared to *Xnr-1* MO-1, was injected into 4-cell stage embryos at the same dose (1mM, 10nl). The 5-mismatch MO-1 elicited only up to 3% left-right reversal even in the left-side injections (Fig. 3D). In addition, the *Xnr-1* 5-mismatch MO-1 did not change the laterality of *Xnr-1* expression in the left LPM (Fig. 5C, D and Table 2). The 5-mismatch *Xnr-1* MO-2, a control oligonucleotide for *Xnr-1* MO-2, also did not cause significant left-right reversal (Fig. 3G).

Injection of Nodal protein into the right side of neurula embryos fully reverses the organs situs

The *Xnr-1* MO injections strongly suggest that unilateral *Xnr-1* expression contributes to left-right orientation. Next, we activated Nodal signaling on either side of the embryo at the neurula stage. By injecting Nodal protein on one side of the flanks of neurula embryos, we examined the possibility that Xnr-1 signaling affects left-right specification after gastrulation. Several hours after injecting Nodal protein mixed with Nile Blue solution into the right flank of the neurula embryo, the embryo was cut into a cross section with a knife at the neural tube stage or early tailbud stage. The staining was localized in the same side, especially in the LPM (Fig. 6A, B). Also in the case of left-side injection, the spot of the

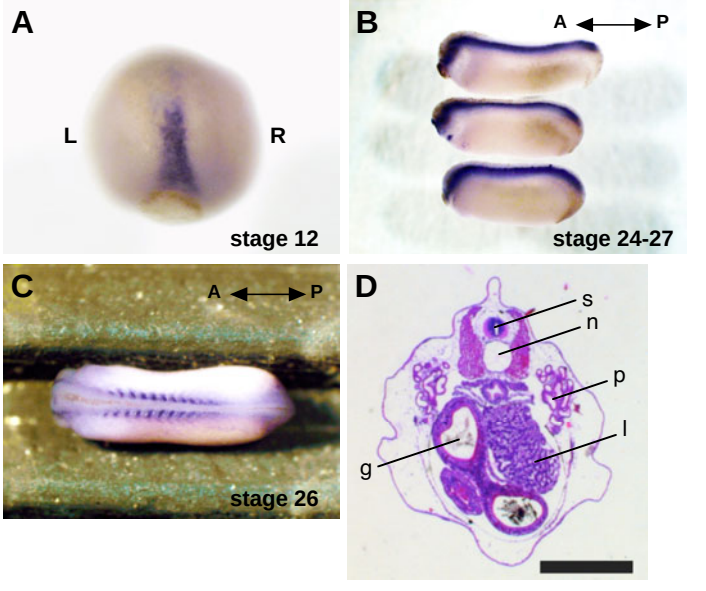
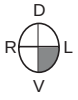


Fig. 4. *Xnr-1* MO does not change the expression of early axial marker genes. (A) *Dorsal expression of* sonic hedgehog *(*shh*) in the gastrula embryo (stage 12).* Xnr-1 *MO-1 injection into two dorsal blastomeres of 4 cell-stage embryos did not change* shh *expression in the organizer region in the majority of the embryos (n=29/33).* **(B)** *The expression of* shh *in the dorsal axial structure of tailbud embryos was also not changed in all of the embryos that had injected with* Xnr-1 *MO-1 into a dorsal blastomere at the 4-cell stage (n=35/35).* **(C)** *The expression of* TGF-β5 *in somites was not affected by the* Xnr-1 *MO-1 injection into a dorsal blastomere of 4-cell embryos (n=22/22).* **(D)** *The cross section of the larva injected with* Xnr-1 *MO-3 is observed to be normal except for the left-right reversal. Cross sections were prepared and observed for stage 43-46 larvae injected with* Xnr-1 *MO-1, 2 or 3 (n=9, 14, 4, respectively). s, spinal cord; n, notochord; p, pronephros; g, gut; l, liver. Scale bar, 0.5 mm.*

TABLE 3

THE EXPRESSION PATTERN OF *XNR-1* IN THE ARCHENTERON ROOF OF TAILBUD EMBRYOS

		Bilateral	Left-alone	Right-alone	Not detected
***Xnr-1* MO-1**		89%	0%	0%	11%
		32/36	0/36	0/36	4/36
		91%	0%	0%	9%
		30/33	0/33	0/33	3/33
***Xnr-1* 5-mismatch MO-1**		79%	0%	0%	21%
		31/39	0/39	0/39	8/39
		83%	0%	0%	17%
		30/36	0/36	0/36	6/36

Injection of *Xnr-1* MO-1 did not suppress the expression of *Xnr-1* in this region, suggesting that initiation or maintenance of the bilateral *Xnr-1* expression in the archenteron roof is free of Xnr-1-dependent mechanism.

dye was confined to the same side during the tailbud stage (Fig. 6C, D). Injection of 2.5-5ng Nodal protein into the right flank of early-late neurula embryos induced left-right reversal of the internal organs in more than 90% of the embryos (Fig. 6E, F and Table 5). To our knowledge, this is the first report of complete left-right reversal of wild type embryos caused by ectopic administration of Nodal protein. In contrast, left-side injection of Nodal protein at the same stage rarely caused *situs inversus* (Fig. 6E and Table 5). The tailbud-stage embryos that had passed the stage of the onset of *Xnr-1* expression in the left LPM did not change the left-right orientation by the Nodal injection. The embryos were cultured for 14 days after injecting the Nodal protein and they grew up normally except for the left-right reversal (Fig. 6G, H). Seventy-six out of 96 injected larvae (79%) had survived for 10 days, while survival ratio of the siblings were 94% (n=171/181).

In order to verify that Nodal protein injected into the flanks really stimulates the Nodal signaling pathway in *Xenopus* neurulae, Nodal and Lefty proteins were co-injected into the left flank. Injection of Lefty protein alone induced high incidence of left-right reversal, suggesting that Xnr-1 signaling was antagonized by the Lefty protein (Table 6). When various concentrations of Nodal solutions were added to the Lefty solution, the incidence of left-right reversal decreased according to the increasing dose of Nodal protein (Table 6). That is, Nodal and Lefty have opposite effects. This result strongly suggests that ectopically administered Nodal protein actually activated Nodal signaling in the LPM.

Right-side injection of Nodal protein induces Xnr-1 *and* pitx2 *in the right LPM*

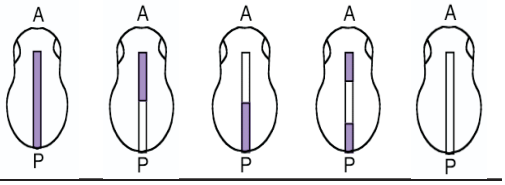
Laterality of the left-handed genes was changed by the ectopically applied Nodal protein. Figure 7 shows the left-right pattern of gene expression when Nodal protein was injected in the right flank under the condition that induced 100% left-right reversal of organ situs. Although a significant alternation in the laterality of *Xnr-1* expression was observed, a considerable proportion of the embryos still kept the original left-handed expression of this gene (Figs. 7B and 7J). *pitx2* was also induced in the right flank in the majority of the injected embryos (Fig. 7G, H and J). In 31% of the embryos showed right-dominant expression pattern («R» or «L<R» in Fig. 7J) after the right-side injection. Meanwhile, left-handed *antivin* expression in the LPM was not altered by the presence of Nodal (Figs. 7F, J). Bilateral paraxial expression of *Xnr-1* at the posterior end was not significantly changed by the Nodal injection (L injection, n=8; R injection, n=4; Fig. 7C, D). Based on these observations, we conclude that Nodal protein was not able to activate the positive feedback loop of *Xnr-1* expression in the right LPM, but that Nodal protein could reorient the left-right axis of the neurula embryos by inducing *pitx2* expression in the right LPM. An additional suggestion is that *Xenopus antivin* must not be indispensable to specify left-right orientation, because complete left-right reversal occurred despite ectopic expression of *antivin* in the right LPM. Right-side injection of Nodal protein did not suppress the axial *antivin* expression (Table 4), suggesting that left-right reversal induced by right-side injection of Nodal was not caused by the breakdown of the midline barrier (see Meno *et al.*, 1998).

Unilateral gene transfer of Xnr-1 *mRNA into one of the LPMs*

Under the favor of the proton sponge effect of polyethyleneimine, 5'-capped full length mRNA of *Xnr-1* was incorporated into the

TABLE 4

THE EXPRESSION PATTERN OF *XENOPUS ANTIVIN* IN THE MIDLINE TISSUES OF TAILBUD EMBRYOS AFTER UNILATERAL MANIPULATION OF NODAL SIGNALING

Injection molecule	Injection point					
***Xnr-1* MO-1**		32%	11%	11%	46%	0%
		9/28	3/28	3/28	13/28	0/28
		16%	0%	81%	3%	0%
		5/32	0/32	26/32	1/32	0/32
Nodal protein	Left side	38%	8%	33%	21%	0%
		9/24	2/24	8/24	5/24	0/24
	Right side	62%	3%	3%	33%	0%
		24/39	1/39	1/39	13/39	0/39
	Uninjected	56%	0%	27%	18%	0%
		12/22	0/22	6/22	4/22	0/22

In the normal embryo, *antivin* expression in the midline structures shows stage-dependent changes along the antero-posterior axis (Cheng *et al.*, 2000, see the control cases in the bottom line), which makes it difficult to estimate the effect of the unilateral injections. However, we notice that neither *Xnr-1* MO-1 nor Nodal protein abolished the expression of axial *antivin*. These results suggest that, in *Xenopus* embryos, maintenance of axial *antivin* is independent of Nodal signaling in the left LPM, which is distinct from the mechanism of axial *lefty* expression in the mouse embryo (Yamamoto *et al.*, 2003).

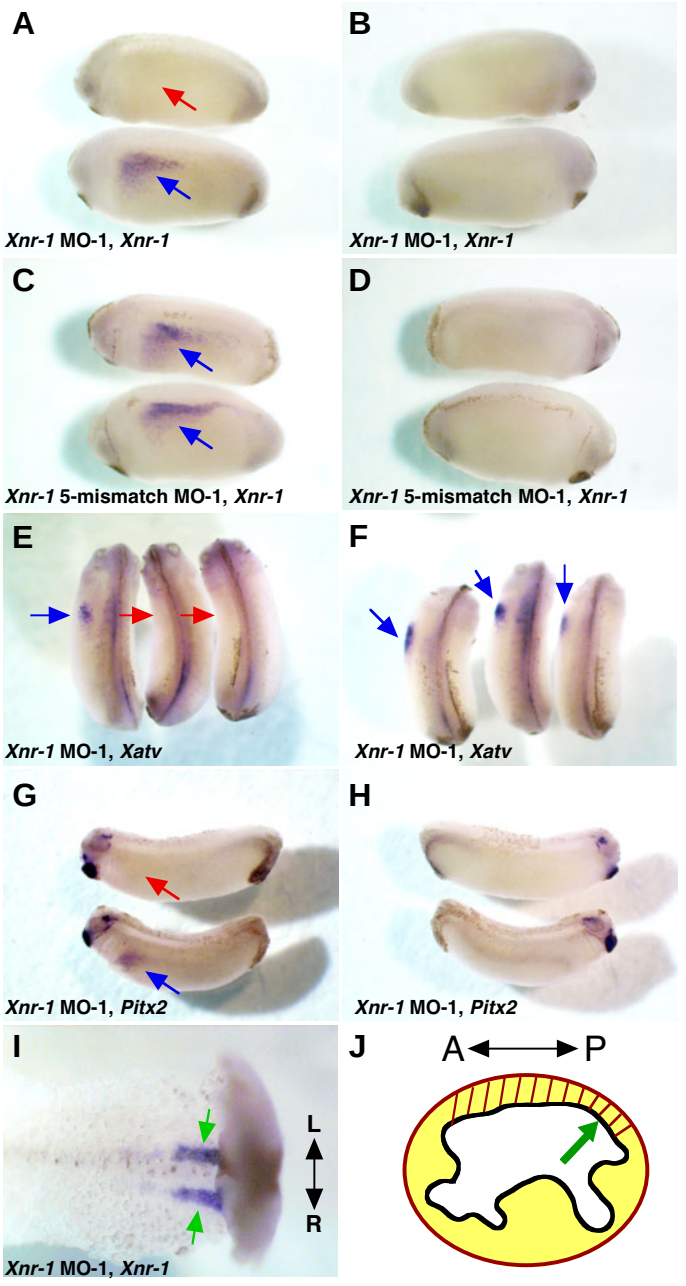


Fig. 5. Alternation of the gene expression pattern of *Xnr-1, Xenopus antivin (Xatv)* and *Xenopus pitx2* after injecting with *Xnr-1* MO-1 (1 mM, 10 nl) into either left or right one of ventral blastomeres at the 4-cell stage. (A) *Tailbud embryo (upper) in which* Xnr-1 *expression in the left LPM has been abolished after* Xnr-1 *MO-1 injection into the* left *blastomere (red arrow). The embryo injected with* Xnr-1 *MO-1 into the* right *blastomere (lower) maintains* Xnr-1 *expression in the left LPM (blue arrow).* **(B)** *Right surface of the embryos shown in (A). No ectopic* Xnr-1 *expression is observed on the right side.* **(C,D)** *Tailbud embryos after the* Xnr-1 *5-mismatch MO-1 injection into the left (upper embryo) or right (lower embryo) blastomere.* Xnr-1 *expression in the left LPM is normal (C, blue arrows) and no ectopic expression is observed in the right LPM (D).* **(E)** *After the* Xnr-1 *MO-1 injection into the ventral* left *blastomere, only one embryo kept* Xatv *expression in the left LPM (blue arrow) and in other embryos the expression was abolished (red arrows). Axial expression of* Xatv *was maintained in the posterior part of the notochord and hypochord or through the full length of these axial structures (for details, see Table 4).* **(F)** Xnr-1 *MO-1 injection into the right blastomere does not interfere with the* Xatv *expression in the left LPM (blue arrows).* **(G,H)** *Expression of* Xenopus pitx2 *in the embryos injected with* Xnr-1 *MO-1 into the ventral left (upper embryo) or ventral right blastomere (lower embryo).* Pitx2 *expression in the left LPM is absent after the left-side injection (G, upper, red arrow), but does not change after the right-side injection (G, lower, blue arrow).* **(I)** *The ventral surface of the archenteron roof of a stage 24/25 embryo after* Xnr-1 *MO-1 injection into the ventral left blastomere. Bilateral posterior paraxial expression of* Xnr-1 *is not changed (green arrows; for details, see Table 3), suggesting that this expression domain is maintained by unknown factor(s) other than* Xnr-1. **(J)** *A line drawing shows the position of the posterior bilateral expression in the sagittal section (green arrow).*

cytoplasm of the LPM cells. Also in this experiment, by the right-side injection, left-right reversal of the internal organs occurred in up to 92% of the injected embryos (Fig. 8B, D). In contrast, gene transfer into the left flank had no significant effect (Fig. 8A, C). Morphology of the injected embryos appeared to be normal except for the organ situs. Injection of polyethyleneimine-based polymer alone into either side of the neurula embryo induced no left-right reversal (Fig. 8C, D). Total incidence of the left-right reversal was not as high as that induced by the Nodal protein. The reason for this may be due to the fact that administration of Nodal protein at high concentration can potentiate Nodal signaling better than the «ectopic protein synthesis» of Xnr-1 in the right LPM.

After the gene transfer in a optimal condition, expression pattern of *pitx2* was investigated. When *Xnr-1* mRNA was incorporated into the left side, expression of *pitx2* kept to the left side (Fig. 8G). On the other hand, the incorporation into the right side induced the *pitx2* expression in the same side for the majority of the embryos. After the right-side injection, 61% of the injected embryos showed bilateral *pitx2* expression and 17% of the embryos showed right-handed *pitx2* expression (Fig. 8H).

For administration of Nodal protein, recombinant mouse Nodal was used. We should note that this manufactured Nodal protein was synthesized in *E. coli* with the expression vector coding only for the mature region of the Nodal protein. Recently Constam group suggested that sugar chain of the Nodal protein potentiates Nodal signaling (Le Good *et al.*, 2005). Lack of the sugar chain in the recombinant Nodal may make it necessary for us to inject a considerable amount of Nodal protein in order to induce more than 90% left-right reversal. In addition, Nodal protein has been reported to be highly unstable *in vitro* (Constam and Robertson, 1999; Le Good *et al.*, 2005). Anyhow, consistency of the results of Nodal injection and *Xnr-1* mRNA incorporation leads us to consider that the right LPM cells have a signaling pathway to respond to Xnr-1 ligand.

Left-side injection of Nodal protein reorients the Xnr-1 *MO-induced left-right reversal*

As described above, injection of *Xnr-1* MOs into the left blastomere of 4-cell stage embryos induced the left-right reversal in 57-77% of the injected embryos.

We examined whether downregulation of Xnr-1 signaling by *Xnr-1* MO could be compensated by the left-side injection of Nodal protein. The embryos that had injected with *Xnr-1* MO-1 into the left ventral blastomere at 4-cell stage were again injected with 10-12ng of Nodal protein into the left LPM at the neurula

stage and cultured until stage 41-42. Sixty percent of the *Xnr-1* MO-injected sibling embryos caused left-right reversal (n=79/132), while the double injection greatly reduced the frequency of the reversal to be 19% (n=18/93; Table 7). Based on this result, we conclude that the main reason of the left-right reversal caused by the *Xnr-1* MO is the lack of Nodal signaling in the left LPM (Fig. 5A, Table 2) and Nodal signaling in the left LPM is thus necessary for normal left-right orientation.

Discussion

Among multiple *Xenopus nodal*-related genes, only *Xnr-1* is expressed in the left LPM. Using two complementary approaches, we investigated the role of the unilateral expression of *Xnr-1*. We found that *Xnr-1* is indispensable for the generation of normal left-right asymmetry and directs the left-right orientation of the internal organs. *Xnr-1* plays critical roles in directing the laterality of the asymmetrically-expressed genes and eventually determining the situs of the internal organs.

Xnr-1 MO seriously downregulated the expression of *Xenopus antivin* and *pitx2* in the left LPM, but did not suppress the posterior bilateral expression of *Xnr-1* (Fig. 5I; Table 3). This result suggests that the posterior bilateral *Xnr-1* expression does not directly induce *antivin* or *pitx2* and that the bilateral *Xnr-1* expression is induced or maintained by a mechanism independent of Xnr-1 signaling.

In the present study, we showed that injection of Nodal protein into the right flank of the neurula embryo induces *Xnr-1* and *pitx2* in the right LPM and completely changes the left-right orientation of the internal organs (Figs. 6F, 8B). Polyethyleneimine-mediated incorporation of *Xnr-1* mRNA into the right LPM cells reproduced the effect of right-side Nodal injection (Fig. 9). These results demonstrate that right LPM is equipped to receive the Nodal (Xnr-1) signal. Coincidentally, mouse, chick and zebrafish somite-stage embryos express *FAST* (*Fox H1*) in both LPMs, which is an essential component of Nodal signaling and thought to be indispensable for *pitx2* activation in the left LPM (Weisberg *et al.*, 1998; Norris and Robertson, 1999; Boggetti *et al.*, 2000; Pogoda *et al.*, 2000; Saijoh *et al.*, 2000; Sirotkin *et al.*, 2000; Hoodless *et al.*, 2001; Shiratori *et al.*, 2001; Norris *et al.*, 2002). Nodal signaling requires EGF-CFC protein, which is a membrane-anchored receptor-associated protein (Schier and Shen, 2000; Minchiotti *et al.*, 2002; Schier, 2003). *EGF-CFC* is also expressed in both LPMs and is involved in the cascade determining left-right asymmetry (Yan *et al.*, 1999; Gaio *et al.*, 1999; Bamford *et al.*, 2000; Schlange *et al.*, 2001). Type I serine/threonine kinase receptor ALK-4 and ALK-7 mediate Nodal signaling (Reissmann *et al.*, 2001). *Xenopus ALK-4 receptor*, which is regarded to play a central role in Xnr-1 signaling (Reissmann *et al.*, 2001; Chen *et al.*, 2004), is also expressed bilaterally in the LPMs and heart fields of tailbud-stage embryos (Chen *et al.*, 2005). Taken together, these data suggest that the absence of Xnr-1 ligand in the right LPM silences the Nodal signaling pathway in the right LPM.

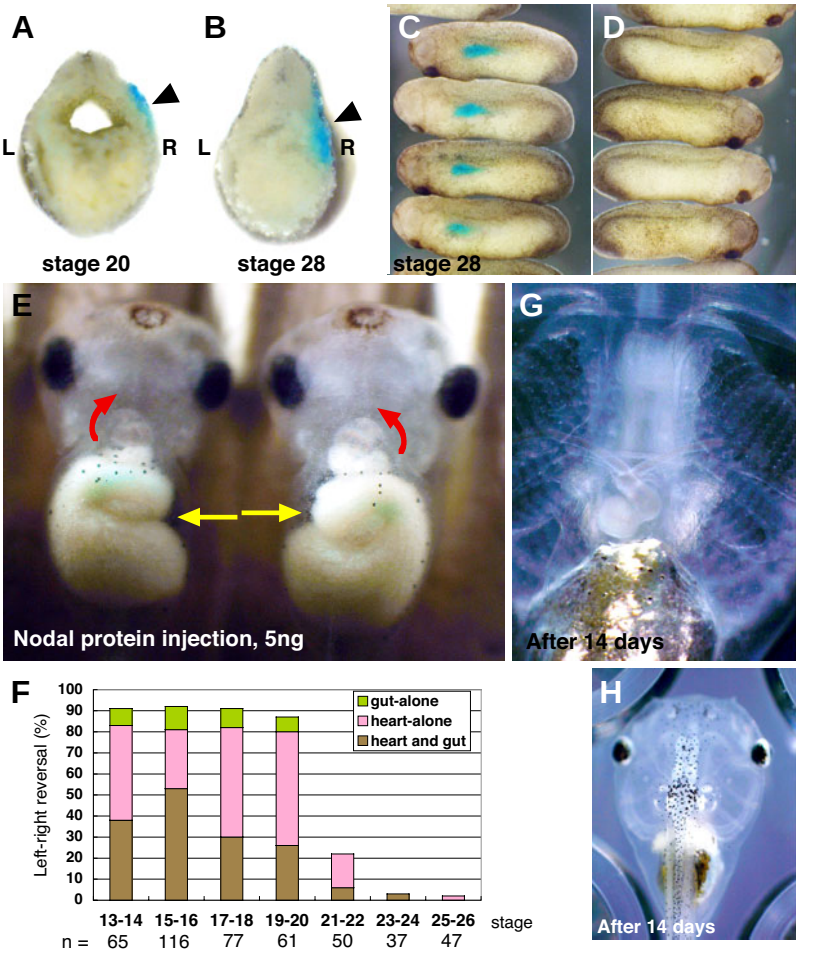


Fig. 6. Injection of Nodal protein into the neurula embryos. (A,B) *Distribution of the Nile Blue solution at stage 20 (A) or at stage 28 (B), after injecting with the mixture of Nodal protein (250μg/ml) and the dye into the right flank at stage 15/16 (midneurula stage). The dye keeps to the periphery of the right side.* **(C,D)** *Left (C) or right (D) surface of tailbud embryos after injecting with 5ng of Nodal protein mixed with Nile Blue (vital dye) into the center of the left LPM at the midneurula stage (stage 15/16). Note that the vital dye is observed in the center of the left LPM and does not leak out of the area.* **(E)** *Left embryo; Stage 42 embryo shows normal situs of the internal organs after injecting with 5ng of Nodal protein into the left LPM. Right embryo; Contrary to the left-side injection, after injecting with Nodal protein into the right LPM at the same stage, the embryo shows left-right reversal in both the heart and gut. Red curved arrows show the direction of blood flow. Yellow arrows indicate the curvature of the gut.* **(F)** *Stage-dependency of the effect of Nodal injection on left-right orientation of the organs. Percentage of the embryos showing left-right reversal after the right-side injection was scored. Nodal injection fully reversed the left-right axis of stage 13-20 neurula embryos, but the early tailbud embryos was not sensitive to the injection.* **(G)** *Magnified view of the inverted heart of a 14-day tadpole after Nodal injection at the neurula stage. Except for left-right orientation, morphology of the heart and gill filaments are normal.* **(H)** *Dorsal view of the 14-day tadpole shown in G. Dorsal axial structures and the sensory organs are normal.*

After the injection of *Xnr-1* MOs or the gene transfer of *Xnr-1* mRNA, morphology of the heart or the gut was minimally affected, demonstrating that Xnr-1 signaling is not centrally involved in organogenesis, with the exception of determining the situs of the organs. Early investigators reported that ectopic overexpression

of *Xnr-1* can induce complete secondary axis in synergy with *noggin* (Lustig *et al*., 1996) or alone (Yamamoto *et al*., 2001). Xnr-1 has been regarded to be an endogenous mesoderm inducer in *Xenopus* (Jones *et al*., 1995; Kofron *et al*., 1999; Agius *et al*., 2000; Chen *et al*., 2004). At first glance, our results seem to be contradictory to the former reports. Here we should note that there are at least 6 *nodal-related* genes in *Xenopus* and the redundancy in the function of multiple Xnr ligands can explain the discrepancy between early reports and the present study. *pitx2* knockout mice and *pitx2* morpholino knockdowns in *Xenopus* revealed that *pitx2* is involved in the morphogenesis of the heart (Gage *et al*., 1999; Lu *et al*., 1999; Kioussi *et al*., 2002a, b; Liu *et al*., 2002; Dagle *et al*., 2003). However, we should note that both in zebrafish and in *Xenopus*, *pitx2* is expressed in early mesoendoderm and prechordal plate mesoderm before asymmetrical *nodal* expression (Essner *et al*., 2000; Faucourt *et al*., 2001). On the other hand, this study changed the laterality of *pitx2* via *Xnr-1* signaling. Suppression of *pitx2* expression only after the neurula stage and intact expression of *pitx2* before this stage can explain why the downregulation of *pitx2* in the left LPM by *Xnr-1* MO (Table 2) did not severely affect the morphology of the heart.

TABLE 5

THE SURVIVAL RATIO AND DOSE-DEPENDENCY OF THE INCIDENCE OF LEFT-RIGHT REVERSAL INDUCED BY HYPODERMIC INJECTIONS OF NODAL PROTEIN INTO THE RIGHT OR LEFT SIDE OF MIDNEURULA EMBRYOS.

	Dose (ng)	Survival of injected embryos	Total incidence of L-R reversal	L-R reversal of both heart and gut	L-R reversal of heart-alone	L-R reversal of gut-alone (%)
Right side	10	86	84	42	29	13
		31/36	26/31	13/31	9/31	4/31
	5	97	92	53	28	11
		116/120	107/116	61/116	33/116	13/116
	2.5	100	100	88	13	0
		24/24	24/24	21/24	3/24	0/24
	1	97	57	6	40	11
		35/36	20/35	2/35	14/35	4/35
	0.01-0.1	96	0	0	0	0
		69/72	0/69	0/69	0/69	0/69
	0.001	97	0	0	0	0
		35/36	0/35	0/35	0/35	0/35
	0	100	0	0	0	0
		63/63	0/63	0/63	0/63	0/63
Left side	10	83	0	0	0	0
		29/35	0/29	0/29	0/29	0/29
	5	91	2	0	2	0
		86/95	2/86	0/86	2/86	0/86
	2.5	100	0	0	0	0
		24/24	0/24	0/24	0/24	0/24
	1	100	0	0	0	0
		24/24	0/24	0/24	0/24	0/24

Both the heart and gut were fully and concordantly reversed by right-side injection, suggesting that Nodal signaling in the LPM controls the orientation of left-right axis of the internal organs.

TABLE 6

CO-INJECTION OF NODAL AND LEFTY PROTEIN INTO THE LEFT FLANK OF *XENOPUS* NEURULAE

Lefty protein	Nodal protein (ng), Left		
	0	1	6
	Upper, %.	Lower, inverted/survived.	
Left, 6ng	57	34	1
	113/198	14/41	1/73

Injection of Lefty alone induced high incidence of left-right reversal, whereas coadministration of Nodal protein restores normal left-right orientation by a dose dependent manner. This result suggests that Lefty blocks Xnr-1 signaling in the left LPM and Nodal restores the Xnr-1 signaling antagonistically.

In *Xenopus* and zebrafish, three *pitx2* isoforms have been identified and the isoforms have different and overlapped expression patterns (Essner *et al*., 2000; Schweickert *et al*., 2000). We observed the downregulation of *pitx2c* in *Xnr-1* MO-injected embryos. When the expression of *pitx2a* and *pitx2b* is not so much affected by *Xnr-1* (Schweickert *et al*., 2000), functional overlapping of *pitx2* isoforms may also prevent the occurrence of the malformation of the internal organs in the *pitx2c*-deficient embryos.

In the present study, we analyzed the laterality of the embryos by the injection of Nodal protein or gene transfer of *Xnr-1* mRNA at the neurula stage, the stage after gastrulation. Thus, we can analyze the role of Nodal signaling in the left LPM separately from the Nodal signaling in earlier embryogenesis before neurulation. This "transient signaling" approach offers a concise method for examining the role of Nodal signaling in a distinct phase of embryogenesis, which is comparable to the analysis of the node-specific *nodal* hypomorph in mice or *southpaw* morphants in zebrafish (Feldman *et al*., 2000; Lowe *et al*., 2001; Saijoh *et al*., 2003; Long *et al*., 2003).

Xnr-1 *MO experiment shed light on the relationships among the three left-handed genes*

When Xnr-1 signaling in the left LPM was inhibited by *Xnr-1* MO, the left-handed expression of *Xnr-1* was downregulated. This observation supports the idea that *Xnr-1* expression in the left LPM initiates an autoregulatory positive feedback loop, analogous to the process of *Xenopus* mesoderm induction (Hyde and Old, 2000; Osada *et al*., 2000) or maintenance of *nodal* expression in the mouse/zebrafish left LPM (Norris and Robertson, 1999; Pogoda *et al*., 2000; Iratni *et al*., 2002; Brennan *et al*., 2002).

Microinjection of the *Xnr-1* MO downregulated the *antivin* expression in the left LPM. Cheng *et al*. (2000) proposed that, in *Xenopus* left LPM, *Xnr-1* induces *antivin* expression and our observations support their idea. On the other hand, microinjection of Nodal protein into the right flank of neurulae did not induce *antivin* expression in the right LPM, raising a possibility that some differential commitment of the two LPMs is responsible for the difference of *antivin*-inducing activity. Alternatively, *antivin* expression may require modest and proper intensity of Xnr-1 signaling and moderate signaling could have induced *antivin* in the right

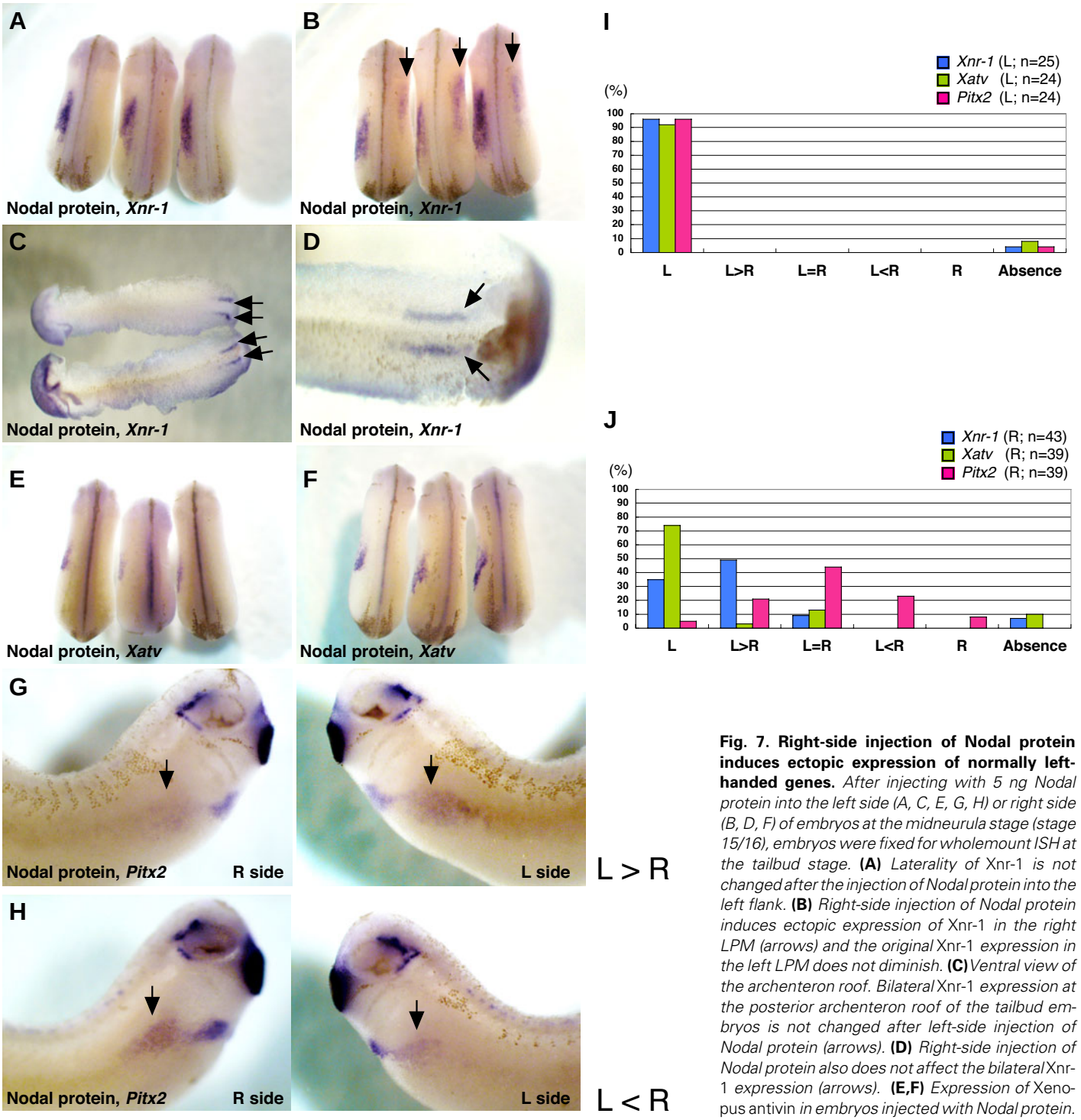


Fig. 7. Right-side injection of Nodal protein induces ectopic expression of normally left-handed genes. *After injecting with 5 ng Nodal protein into the left side (A, C, E, G, H) or right side (B, D, F) of embryos at the midneurula stage (stage 15/16), embryos were fixed for wholemount ISH at the tailbud stage.* **(A)** *Laterality of* Xnr-1 *is not changed after the injection of Nodal protein into the left flank.* **(B)** *Right-side injection of Nodal protein induces ectopic expression of* Xnr-1 *in the right LPM (arrows) and the original* Xnr-1 *expression in the left LPM does not diminish.* **(C)** *Ventral view of the archenteron roof. Bilateral* Xnr-1 *expression at the posterior archenteron roof of the tailbud embryos is not changed after left-side injection of Nodal protein (arrows).* **(D)** *Right-side injection of Nodal protein also does not affect the bilateral* Xnr-1 *expression (arrows).* **(E,F)** *Expression of* Xenopus antivin *in embryos injected with Nodal protein. Laterality of* antivin *expression is not changed either by the left (E) or right (F) injection of Nodal.* **(G,H)** *Injection of Nodal protein into the right flank induces* Xenopus pitx2 *expression in the right flank. An embryo in G shows the case of bilateral but left-dominant expression of* pitx2 *in LPMs (corresponding to «L>R» in Fig. 7J), whereas the one in H shows the right-dominant case (correspoinding to «L<R» in Fig. 7J). Bilateral expression of* pitx2 *at the posterior end of the eyes is not changed by the Nodal injection.* **(I,J)** *Effects of the injection of Nodal protein into the left (I) or right (J) flank of the midneurula embryos. Laterality of the normally left-handed* Xnr-1, Xenopus antivin (Xatv) *and* Xenopus pitx2 *was assessed for the embryos at stage 24-26 (*Xnr-1, Xatv*) or stage 28-30 (*pitx2*). When Nodal was injected on the left side,* Xnr-1, antivin *and* pitx2 *kept the original left-handed expression. On the other hand, after the right-side injection of Nodal, both* Xnr-1 *and* pitx2 *were induced in the right LPM (*Xnr-1, *58%, n=25/43;* pitx2, *87%, n=34/39), whereas, in 74% of the embryos injected with Nodal (n=29/39),* antivin *kept the original left-handed expression.*

TABLE 7

COMPARISON OF THE INCIDENCE OF LEFT-RIGHT REVERSAL BETWEEN THE EMBRYOS WITH *XNR-1* MO-1 INJECTION INTO THE LEFT BLASTOMERE AND THE SIBLINGS WITH SEQUENTIAL INJECTION OF *XNR-1* MO-1 AND NODAL PROTEIN INTO THE LEFT BLASTOMERE AND LATER IN THE LEFT FLANK

Xnr-1 MO-1 1mM, 10nl	Nodal protein (ng), Left	
	0	10-12
	Upper, %.	Lower, inverted/survived.
D R L V	60	19
	79/132	18/93

Injection of Nodal protein into the left flank of neurulae restores normal organ situs out of the randomization of left-right orientation potentially caused by *Xnr-1* MO-1.

LPM. Anyhow, we should note that even when *antivin* was still expressed only in the left LPM after the right-side Nodal injection, left-right asymmetry of the internal organs was completely inverted (Figs. 7F, 8B). This result suggests that *antivin* is not the initiator of left-right orientation, but only acts as a regulator of Nodal signaling in *Xenopus* embryos. Our results with Nodal injection weaken the possibility that *pitx2* induces *antivin* in the left LPM of *Xenopus* embryos, because in the embryos injected with Nodal, laterality of the *pitx2* and *antivin* takes opposite side.

Implications of the present study for axial* antivin *expression

Early investigators suggest that, in *Xenopus* embryos, midline tissues act as a barrier to separate the left and right humoral signals and confine the *Xnr-1* expression in the left LPM during the process of left-right specification (Danos and Yost, 1996; Lohr *et al*., 1997; Lohr *et al*., 1998). In mouse embryos, *lefty-1* is expressed strongly in the left half of the floor plate and notochord and weakly in the left LPM (Meno *et al*., 1996; Meno *et al*., 1997; Meno *et al*., 1998). *lefty-1*-null mice show left isomerism in the internal organs (Meno *et al*., 1998). From these results, Lefty (Antivin) is regarded to act as a midline barrier in order to prevent the diffusive Nodal ligand from functioning on the opposite side (*i.e.*, the right side). Recent studies suggest that, in mouse embryos, *nodal* expressed in the node induces *nodal* expression in the left LPM and the two *nodal* expressing domains cooperatively induce the axial *lefty* expression (Brennan *et al*., 2002; Saijoh *et al*., 2003; Yamamoto *et al*., 2003).

In *Xenopus* embryos, it is noteworthy that axial expression of *Xenopus antivin* begins far before the onset of left expression of *Xnr-1*, which is contrary to the case of mouse embryos (Cheng *et al*., 2000). Therefore, we can not suppose that unilateral *Xnr-1* expression induces the axial *antivin* expression. The present study supports this prediction, because *Xnr-1* MO did not affect the axial expression of *antivin* (Table 4). We propose that, in *Xenopus* embryos, axial *antivin* is initiated and maintained by a mechanism distinct from that of mouse embryos. The "axial *antivin*-inducing factor" may be a Nodal-related ligand other than Xnr-1. It might be supposed that one candidate is *Xnr-4*, which is expressed in the notochord and ventral neural tube of the midneurula embryo (Joseph and Melton, 1997), resembling the expression pattern of axial *antivin*. The redundancy of Nodal-related ligands in *Xenopus* embryos may evolve a novel mechanism for the axial *antivin* expression different from that of the rodents.

Relationships between posterior bilateral* Xnr-1 *expression and the expression in the left LPM

In mouse somite-stage embryos, Nodal protein that is secreted in the peripheral margin of the node is supposed to be delivered toward the left side by the nodal flow generated by the rotational movement of monocilia in the node (Nonaka *et al*., 1998; Nonaka *et al*., 2002). Through the leftward nodal flow, Nodal ligand is supposed to travel into the left LPM and induce *nodal* expression in the left LPM (McGrath *et al*., 2003; Yamamoto *et al*., 2003; Watanabe *et al*., 2003; Cartwright *et al*., 2004). Yost and colleagues found that in zebrafish, *Xenopus* and chick embryos, mRNA of *left-right dynein* and rod-like structures resembling monocilia are sharply localized in the late organizer region of each of these animals (Essner *et al*., 2002; Essner *et al*., 2005). Recently, Okada *et al.* (2005) reported that rabbit and medakafish embryos also exhibit a leftward fluid flow in their organizer regions, strongly suggesting that nodal flow is a conserved mechanism among other vertebrates. Moreover, Hirokawa group discovered that, FGF signalling triggers secretion of membrane-sheathed objects 0.3-5 μm in diameter termed 'nodal vesicular parcels' (NVPs) that carry Sonic hedgehog and retinoic acid in the mouse embryo. These NVPs are transported leftward by the fluid flow and eventually fragment close to the left wall of the ventral node (Tanaka *et al.,* 2005).

In *Xenopus*, the expression domain of *left-right dynein* and the distribution of monocilia detected by anti-acetylated tubulin appear to cover the area of posterior bilateral *Xnr-1* expression (Essner *et al*., 2002). However, it has not yet been examined whether there is left-right asymmetrical nodal flow within the archenteron of *Xenopus* neurula embryos. Even if the nodal flow exists in the archenteron roof, it seems difficult to suppose that such flow is stably left-right asymmetrical in the compact spherical space of the archenteron.

Nevertheless, the hypothesis of leftward nodal flow in the *Xenopus* neurula embryo is attractive, because this hypothetical flow can easily explain the generation of posterior left *Xnr-1* expression. Posterior bilateral *Xnr-1* expression begins at stage 18 and continues until stage 30 or later, while *Xnr-1* expression in the left LPM begins at stage 19 (Lowe *et al*., 1996; our observation). That is to say, posterior bilateral *Xnr-1* expression precedes the unilateral expression. This study revealed that the posterior bilateral expression is not affected by *Xnr-1* MO injection (Fig. 5I, Table 3), while unilateral *Xnr-1* expression is severely affected by the *Xnr-1* MO (Fig. 5A, B, Table 2). This observation is reminiscent of the report that posterior bilateral *southpaw* expression in the zebrafish embryo is not affected by the *southpaw* MO (Long *et al*., 2003). It is possible that some secretory factor(s) induces the posterior bilateral *Xnr-1* expression, but the physiological role of this bilateral expression is unknown. Because Nodal is a diffusive secretory signaling molecule (Meno *et al*., 1999; Meno *et al*., 2001; Sakuma *et al*., 2002), it is reasonable to suppose that the posterior bilateral *Xnr-1* expression supplies the Xnr-1 ligand that diffuses toward the left LPM to induce *Xnr-1* expression by an autoregulatory feedback mechanism. Actually, we show here that right LPM can transduce a Nodal (Xnr-1) signal (Figs. 7, 8), which suggests that lack of Xnr-1 ligand silences the *nodal-pitx2* cas-

cade in the right side. Xnr-1 ligand secreted in the dorsal posterior-end may not flow into the right LPM.

Kupffer's vesicle is a transient organ that is thought to be required to generate zebrafish left-right asymmetry. Kupffer's vesicle has monocilia, expresses *left-right dynein* and generates nodal flow in zebrafish embryos (Essner *et al.*, 2002; Amack and Yost, 2004; Essner *et al.*, 2005). Furthermore, zebrafish *nodal*-related gene *southpaw* is expressed in adjacent to the Kupffer's vesicle. *southpaw* is expressed bilaterally in posterior paraxial mesoderm at first and then the expression is biased to the left side and induces *cyclops* in the left LPM. Charon, a member of Cerberus/Dan family of secreted glycoproteins is reported to regulate the asymmetrical expression of *southpaw* via direct association of Charon with Southpaw around the Kupffer's vesicle (Hashimoto *et al.*, 2004). Thus, this domain is regarded to be the organizing center of the left-right axis in the zebrafish embryo. Recently, mouse Cerberus-like 2 (Cerl-2; Dante) was identified and this molecule also plays a key role in restricting the Nodal signaling toward the left side by preventing its activity in the right side (Marques *et al.*, 2004). In *Xenopus* embryos, expression of the gene coding for a member of Cerberus/Dan family such as Charon or Cerl-2 at the tail region has not yet been reported. We should consider the possibility that asymmetrical expression of Cerberus/Dan-related secretory protein in the posterior right side may absorb the Xnr-1 ligand and silence the right-handed propagation of *Xnr-1* expression wave.

Conclusions

As above stated, resumption of *Xnr-1* transcription as the posterior bilateral expression appears to play a pivotal role which connects the early phase of left-right specification before neurula stage and the later phase of left-right differentiation including the *Xnr-1-pitx2* pathway. Posterior bilateral expression of *Xnr-1* covers the inner surface of the archenteron in late neurula embryos and it is thus probable that the neurula archenteron is a pool for left-right axis determination. As a first step to test this idea, we should perform sampling and profiling of the growth factors secreted into the archenteron and visualizing the fluid flow inside the archenteron.

In conclusion, we investigated the role of left-handed *Xnr-1* expression using *Xnr-1* MO and found that the unilateral expression of *Xnr-1* is indispensable for normal left-right orientation. *Xnr-1* acts as a "left-indicator" per se and

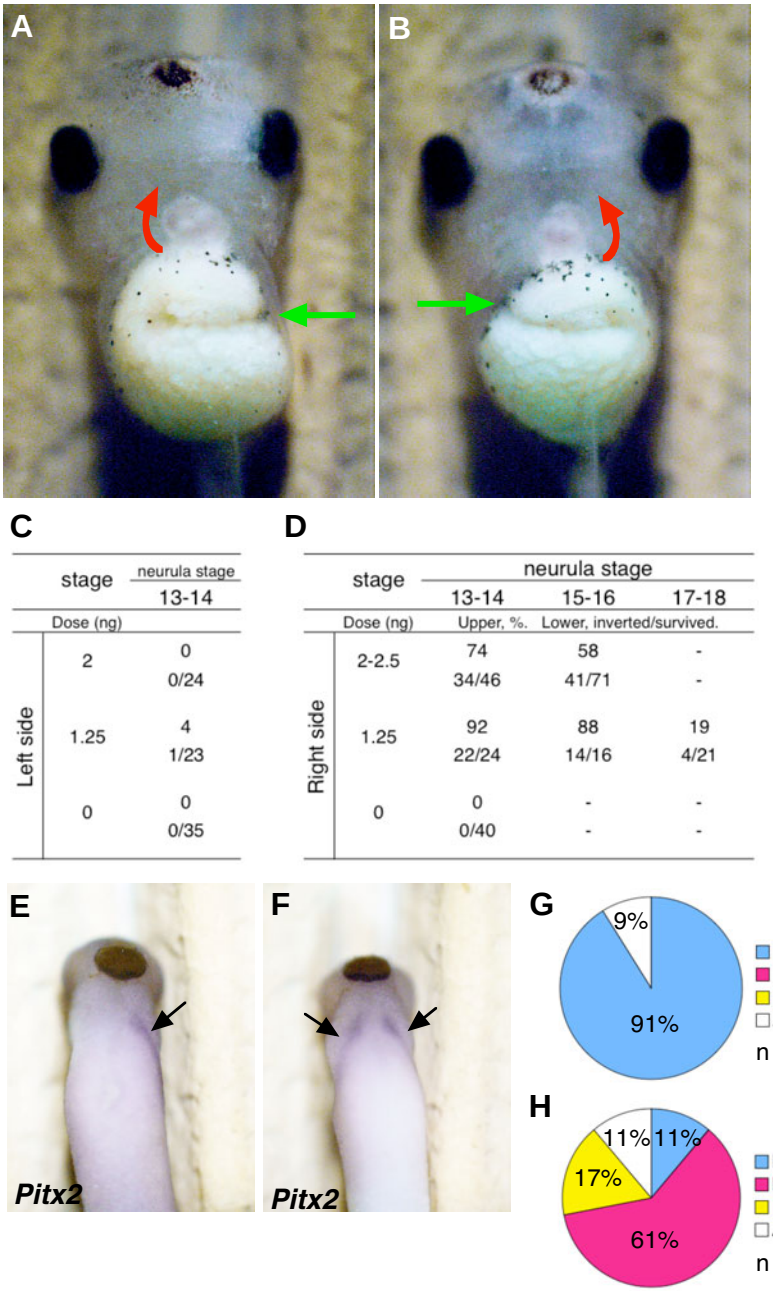


Fig. 8. Incorporation of *Xnr-1* mRNA into the cells of lateral plate mesoderm of the neurula embryo. (A,B) *Using polyethyleneimine-based polymer as a carrier, 2ng of the mRNA was injected into the left (A) or right (B) side of the early neurula (stage 13/14) embryo. An embryo in A shows normal situs, while the one in B shows left-right reversal in both the heart and gut. Note that outer shapes of the embryos are normal. Red curved arrows show the direction of blood flow. Green arrows indicate the curvature of the gut.* **(C,D)** *The incidence of left-right reversal in the embryos that were injected with the mixture of* Xnr-1 *mRNA and polyethyleneimine. The left-side injection did not induce significant left-right reversal (C), while the right-side injection induced up to 92% reversal (D).* **(E,F)** *Expression of* pitx2 *after injecting with 2.5ng* Xnr-1 *mRNA into the left side (E) or right side (F) of the early neurula embryos. An embryo in (E) shows normal left-handed* pitx2 *expression (ventral view), while the one in (F) shows bilateral* pitx2 *expression in the LPMs.* **(G,H)** *Classification of the expression pattern of* pitx2 *after the gene transfer of* Xnr-1 *mRNA. In (G), the left-side injection did not affect the laterality of* pitx2 *expression in the LPM. In (H), the right-side injection induced* pitx2 *expression in the right LPM for the majority of the injected embryos.*

minimally affects the shapes of the organs. Left-side injection of Nodal protein into the precedently *Xnr-1* MO-injected embryos restores normal situs out of randomization of the left-right axis by the MO, demonstrating the unique role of Xnr-1 signaling in left-right axis determination. Activation of Xnr-1 signaling in the right LPM completely inverted the left-right axis of the internal organs, which complements the *Xnr-1* MO experiment.

Materials and Methods

Xenopus *eggs and microinjection*

Fertilized *Xenopus laevis* eggs were obtained from a mating pair of adults stimulated by injection 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. Developmental stages were identified according to the normal developmental table of Nieuwkoop and Faber (1967).

To inhibit endogenous *Xenopus nodal related-1* (*Xnr-1*)-specific signaling, 5nl or 10 nl of 1mM antisense morpholino oligonucleotide-1 (MO-1) targeting start codon region of *Xnr-1* (5'-ACA GGA CTG CTG TCA GAA ATG CCA T-3'; designed to hybridize to the CDS 48..72 of GenBank No. U29447 [CDS 48..1268]; Gene Tools, LLC, Philomath, OR) was injected into one or two blastomeres of 4 cell-stage embryos. To verify the specific effect of the MO-1 on Xnr-1 signaling, *Xnr-1* MO-2 targeting the splicing region between the first exon (116..123; AF410903S2) and first intron (124..140; AF410903S2) of *Xnr-1* (5'-ACA GAT AAT TTA CTC ACT TTT TGC A-3'; designed to hybridize to the 116..140th sequence of GenBank No. AF410903S2) and *Xnr-1* MO-3 targeting 5'-UTR of *Xnr-1* (5'-CTT GCA CTG CTG ATC TCT CTT TCA G-3'; designed to hybridize to the 22..44th sequence of GenBank No. XLU29447 [coding region: 48..1268]) were also tested. The *Xnr-1* MO-2 and *Xnr-1* MO-3 were labeled with FITC for tracing the offspring of the injected cells. A control MO that had a five bp mismatch in sequence compared with that of original *Xnr-1* MO-1 sequence (5'-ACA CGA CTC CTG TGA GAA TTG CGA T-3') was injected into the cleavage-stage embryos at the same dose. *Xnr-1* 5-mismatch MO-2 (5'-AGA CAT AAT TTA GTC AGT TTT TGG A-3') was also used as another control oligonucleotide.

The sequence of *Xnr-1* MO-1 is not expected to hybridize to *Xnr-2* (GenBank No. U29448), *Xnr-3* (GenBank No. U25993), *Xnr-4* (GenBank No. U79162), *Xnr-5* (GenBank No. AB038133) or *Xnr-6* (GenBank No. AB038134), since the *Xnr-1* MO has, at a minimum, a ten, eight, seven, eleven, or nine bp mismatch, respectively, with the sequence of each of these *Xnrs*. Similar multiple alignment analysis revealed that the *Xnr-1* MO-2 or MO-3 has no complementary sequence for *Xnr-2*, *Xnr-3*, *Xnr-4*, *Xnr-5* or *Xnr-6* mRNA.

Immediately after microinjection, the embryos were placed in saline containing 4% Ficoll (ICN Biomedicals Inc., Irvine, CA), cultured overnight and then transferred into saline without Ficoll and cultured further at 18-26ºC until they reached stage 41-42. At this stage, left-right orientation of the heart and visceral organs was examined and the incidence of left-right reversal in the injected embryos was scored according to the criteria as previously described (Toyoizumi *et al.*, 1997, 2000; Mogi *et al.*, 2003). To more precisely judge the gut asymmetry, the laterality of the ventral pancreas was also checked. The embryos were scored for morphogenetic pattering along the antero-posterior and dorso-ventral axes using the dorso-anterior index (DAI; Kao and Elinson, 1988).

For microinjection at the neurula stage, embryos were de-chorionized and transferred into a Terasaki plastic plate (Sumitomo Bakelite Co., Tokyo, Japan) filled with saline. The embryos were injected hypodermically in the center of their left or right flanks with 5 nl, 25 nl or 50 nl of the following protein solutions using an electric microinjector ('Nanoject', Drummond Scientific Co., Broomall, PA). To verify the injection site at a later stage, the protein solutions were mixed with 10% Nile Blue vital dye solution (Wako Co., Tokyo, Japan) at a ratio of 10:1 in all cases. The proteins were injected primarily at concentrations of 0.2-200 μg/mL Nodal protein (recombinant mouse Nodal, R & D Systems Inc., Minneapolis, MN). According to the manufacturer's information, only the sequences coding for the mature region of mouse Nodal ligand were subcloned into the expression vector. Thus, because the pro-domain was eliminated from the recombinant Nodal, the Nodal protein in this report consists only of mature ligands. For control experiments, 5 nl or 25 nl of 1% BSA solution (bovine serum albumin fraction V, Sigma, St. Louis, MO) was injected. Lefty protein (recombinant mouse Lefty-1, R & D Systems) was also used at a concentration of 250 μg/mL in part of the injection experiments.

After microinjection, the embryos were transferred into individual wells of 24-well test plates (Iwaki Glass Co., Tokyo, Japan) and cultured at 18-26ºC until they reached stage 41-42.

In vitro *translation*

Xnr-1 mRNA was transcribed *in vitro* using T3 polymerase and mMESSAGE mMACHINE *in vitro* transcription kit (Ambion co.) at 37ºC for two hours. The precipitate was dissolved in RNase-free water and then used for *in vitro* translation.

In order to prove that *Xnr-1* MO-1 surely inhibits the translation of Xnr-1 protein, fluorescent-labeled Lysine was incorporated into the product during translation. One microgram of 5'-capped *Xnr-1* mRNA was denatured at 65ºC for 3 min and the 1 mM solution of *Xnr-1* MO-1 or 5-bp mismatch *Xnr-1* MO-1 was added to the mRNA solution and then the mixture was incubated at room temperature for 30 min. Using rabbit Reticulocyte Lysate system (Promega co.) in combination with FluoroTect™ Green-Lys *in vitro* Translation Labeling System (Promega co.), fluorescent-labeled Xnr-1 protein was synthesized *in vitro* at 30ºC for 90min. The fluorescent Xnr-1 protein was dissolved in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCI pH6.8, 2% SDS, 0.004% bromophenol blue, 10% glycerol, 100 mM dithiothreitol) and electrophoresed on SDS-polyacrylamide gels at constant current of 20 mA. Immediately after the electrophoresis, the fluorescent product was scanned and detected with the molecular imager (BioRad FX) in order to examine whether *Xnr-1* MO-1 inhibits the protein synthesis of *Xnr-1*. As a positive control, *Xnr-1* mRNA free of *Xnr-1* MO-1 was used for the template.

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 a gift from Dr. Christopher V. E. Wright (Vanderbilt University). cDNA of *Xenopus Pitx2c* (*Pitx2*) was a gift from Dr. Juan Carlos Izpisúa Belmonte (The Salk Institute). cDNA of *Xenopus TGF-β5* was a gift from Douglas A. Melton (Harvard University). *Xenopus antivin* is 99% identical to *Xlefty-B* and 92% identical to *Xlefty-A*. Based on the identical spatio-temporal expression pattern and highly homologous sequences, Branford *et al.* (2000) concluded that *Xlefty-A* and *Xlefty-B* are alleles of the same gene, *Xlefty*.

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 staining. The expression patterns of *Xnr-1*, *Xatv* and *Pitx2* genes were examined by whole-mount *in situ* hybridization according to Harland's methods (1991) with slight modifications. Antisense RNA probes and control sense probes were labeled with digoxigenin. Stained embryos were washed with PBS- containing 0.1% Tween 20, photographed and stored in 50% glycerol at 4ºC. Part of the embryos were photographed after penetrating with 50% glycerol solution. We attached importance to the area and intensity of the expression in the LPM as in our previous papers (Toyoizumi *et al.,* 2000; Mogi *et al.,* 2003). For individual specimen, both LPMs were carefully checked and scored for the laterality of gene expression.

Gene transfer of Xnr-1 *mRNA into lateral plate mesoderm cells*

Xnr-1 mRNA was synthesized *in vitro* at 37ºC for 60 minutes and capped

at 5' end using mMESSAGE mMACHINE™ (Ambion Co.) and T3 polymerase. Stock solution of *Xnr-1* mRNA was dissolved in Marc's Modified Ringers (MMR, 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, 0.1 mM EDTA, pH7.4) containing 5% glucose to the concentration of 850μg/ml. Then the mRNA solution was mixed with *in vivo*-jet PEI™ solution (Polyscience Co.; Cat. No. 201-10) and stirred by pipetting. After incubating the mixture at room temperature for 15 minutes, the mixture was again stirred and prepared for microinjection. Final concentration of *Xnr-1* mRNA was 50 μg/ml. *Xnr-1* mRNA-jet PEI mixture solution (10-50 nl) was injected into the right or left flank of the early-late neurula embryos. Protocol of gene transfer using jet PEI solution requires 12-48 hour for efficient incorporation of the mRNA. Thus, the embryos injected with *Xnr-1* mRNA-jet PEI mixture were incubated at low temperature (14-15ºC) to prolong the neurula stage for long time. After overnight incubation at the low temperature, the embryos were cultured at 18-24ºC.

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