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Original Article

Xerl, a novel CNS-specific secretory protein, establishes the boundary between neural plate and neural crest

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ABSTRACT A novel gene, *Xerl,* has been found as a CNS-specific gene encoding a secretory protein. In order to clarify a function of Xerl, we first examined *Xerl*-expressing areas during early development. Comparison with *XlSox-2*-positive neural plate and *ADAM13*-positive neural crest showed that *Xerl* expression was limited within the neural plate area. Microinjection of *Xerl* mRNA into 2- or 4-cell stage embryos indicated that *Xerl* overexpression caused the regional expansion of *XlSox-2*- and *NCAM*-positive neural plate, which was concomitant with the outer shift of *ADAM13*-positive region. The *Xerl* injection resulted in incomplete neural closure because of the local overproduction of the neuroepithelium. In contrast, loss of function analysis of Xerl indicated that Xerl inhibition caused the ectopic differentiation of neural crest cells. In the conjugation experiment using *chordin*-injected animal caps, *Xerl* promoted chordin-induced *XlSox-2* expression, whereas Xerl inhibition caused *ADAM13* expression even in the injection with a high dose of chordin. Animal cap assays also showed that *Xerl* expression was induced by chordin. In the functional analysis using truncated forms of Xerl, Xerl ΔL (lacking LNS domain) worked as a dominant negative form that induced the overproduction of neural crest cells. These results suggest that Xerl is involved in the boundary formation of the neural plate through exclusion of neural crest cell differentiation.

KEY WORDS: *Xenopus, Xerl, neural plate, neural crest*

Introduction

Vertebrate nervous system consists of central nervous system (CNS) and peripheral nervous system (PNS). The first determination of each fate begins at the end of gastrulation. Vertebrate ectoderm is divided into three sets of cells, neural plate, epidermis and neural crest cells, and finally neural plate cells make CNS and also neural crest cells make PNS (Le Douarin, 1982, Hall and Hörstadius, 1988).

In *Xenopus*, prospective neural crest cells can be identified at the late gastrula stage (stage 12) by their expression of the zinc finger transcription factors, *Xsnail* and *Xslug* (Mayor *et al*., 1995, Mancilla and Mayor, 1996). These expressions are initially restricted to the crest-forming boundary of the neural plate. Classical experiment showed that medial archenteron roof was grafted into the blastocoel of amphibian embryos, and competent ectoderm could be induced to form both neural plate and neural crest (Raven and Kloos, 1945). In *Xenopus* embryo, several models explain the neural crest determination. In one model, Raven and Kloos (1945) have postulated the existence of a combined CNS/neural crest "evocator" molecule that is produced at different concentrations across the archenteron roof. Now this model may be explained that BMP receptor (BMPR) activities make a threshold between neural plate and neural crest induction (Mancilla and Mayor, 1996, Marchant *et al*., 1998). Some neuron-inducible molecules derived from dorsal lip are BMP inhibitors like noggin (Lamb *et al*., 1993), chordin (Sasai *et al*., 1994) and follistatin (Fainsod *et al*., 1997). All of these BMP inhibitors are secretory proteins. It has been shown that low doses (pico gram order) of dominant negative BMPR or low doses of chordin can induce gene expression of neural crest-specific genes, whereas high doses (nano gram order) of these molecules can induce neural plate genes (LaBonne and Bronner-Fraser, 1998, Marchant *et al.*, 1998,). However, it is still unknown how secretory inhibitors make such a threshold. If this is the case, how does BMP determine sharp threshold between neural plate and neural crest? There should be a mechanism stocking high level BMP inhibitors in the

Abbreviations used in this paper: CNS, central nervous system; ELR, EGF-like repeat; LNS, laminin A, neurexins and sex hormone binding globulins; PNS, peripheral nervous system.

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CNS or keeping the intermediate activity of BMP inhibitors in the PNS. There is a model about the neural crest development that the encountering between the intermediate level of BMP inhibitor and the neural crest-specific inducing signal causes neural crest cell specification, which have proposed by LaBonne and Bronner-Fraser (1998) and Marchant *et al.* (1998).

Expected property of the postulated factor may be an extracellular protein and to express at specific CNS. Our previous report has shown that a novel *Xenopus* gene, *Xerl*, was expressed in CNS from neurula to early tadpole stage (Kuriyama *et al*., 2000). The prospective structure of Xerl contains EGF-like repeats (ELRs) and laminin-G-like domain including cell attachment sequence, RGD. ELRs also exist in matrix molecules such as fibronectin (Ruoslahti *et al*., 1984) and signal controlling molecules such as Notch (Artavanis-Tsakonas *et al*., 1983), Delta (Fehon *et al*., 1990). Recently, laminin-G domain was found in a large number of proteins like laminin A, neurexins and sex hormone binding globulins, so this domain is called LNS domain (Missler and Südhof, 1998). ELR-containing proteins such as Slit (Rothberg *et al.*, 1990) and Crumbs (Tepass *et al*., 1990) also have the LNS domain. Many proteins with ELRs and LNS domain bind to cell surface receptors, suggesting that the LNS domain is cell-surface recognition elements (Missler and Südhof, 1998). Therefore, Xerl may function as an extracellular protein during neurogenesis of CNS. Here we examine the possibility that Xerl is involved in the boundary formation between neural plate and neural crest.

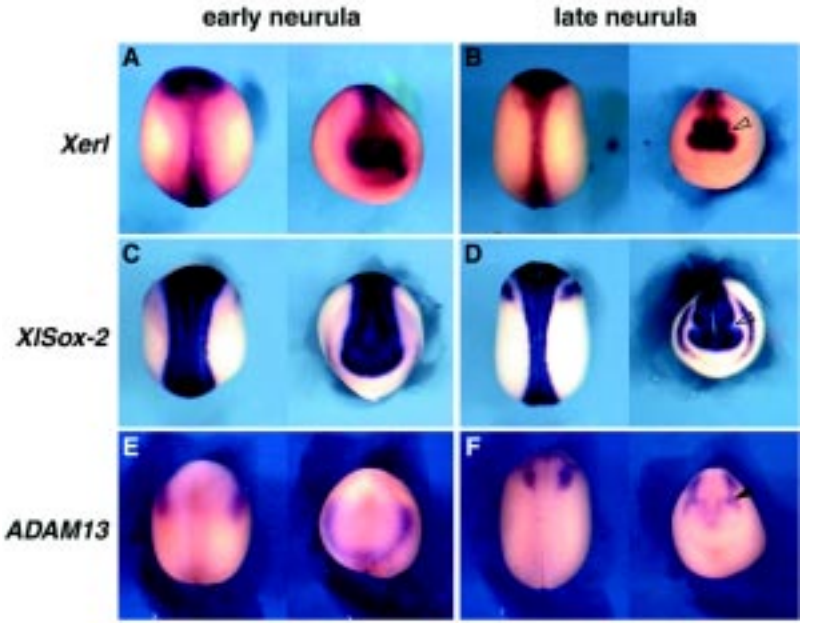

Fig. 1. Comparison of the gene expression patterns of *Xerl*, *XlSox-2* and *ADAM13*. *(A,C,E) Embryos before neural fold closure (st.15). The left is a dorsal view and the right is an anterior view for each picture. (B,D,F) Embryos after neural fold closure (st.20). The left is a dorsal and the right is also an anterior view.* **(A)** Xerl *expression at the neural plate stage.* Xerl *expression was seen in the anterior neural plate and the posterior neural groove.* **(B)** Xerl *expression after neural fold closure. The opened arrowhead indicates the narrow region of gene expression between diencephalon and midbrain.* **(C)** XlSox-2 *expression at the neural plate stage.* XlSox-2 *expression was wider than* Xerl *expression.* **(D)** XlSox-2 *expression after neural fold closure. The opened arrowhead indicates the diencephalon/midbrain boundary.* **(E)** ADAM13 *expression at the neural plate stage.* ADAM13 *expression was complementary to* Xerl *and* XlSox-2. **(F)** ADAM13 *expression after neural fold closure. The arrowhead indicates the diencephalon/midbrain boundary.*

Results

Xerl expression in the Central Nervous System

In our previous report, we showed that *Xerl* was expressed in eye and brain (Kuriyama *et al*., 2000). In order to know Xerl function, we first examined the expression pattern of *Xerl* in details during neurogenesis. Before neural folding, *Xerl* expression was seen in the neural groove along the dorsal midline (Fig. 1A, left) and the inner edge of the anterior neuroectoderm (Fig. 1A, right). As neural folding proceeding, *Xerl* expression was also seen in the trunk neural tube (Fig. 1B, left). In addition, anterior expression of *Xerl* was seen in midbrain, diencephalon, and eye corresponding to the future neural retina. The boundary between midbrain and diencephalon was indicated as a narrow expression of *Xerl* (Fig. 1B, right, opened arrowhead). To confirm *Xerl* expressed only in CNS, we compared *Xerl* expression with *XlSox-2* expression at each corresponding stage (Fig. 1 C,D). *XlSox-2* is general neural plate marker, expressed in the neural plate from the end of gastrulation (Mizuseki *et al*., 1998). Our observation showed that *Xerl* expression occurred within the *XlSox-2*-positive area (Fig. 1 A,C). This relationship between *Xerl* and *XlSox-2* was more obvious by comparison of gene expression at the narrow region between midbrain and diencephalon (Fig. 1 B,D, right, opened arrowhead).

To recognize the neural crest region, we examined *ADAM13* expression at same stages. *ADAM13* is metalloproteinase/disintegrin family gene expressing in cephalic neural crest cells (Alfandari *et al*., 1997, Marchant *et al.*, 1998, Cousin *et al.*, 2000). *ADAM13* expression was seen in the outer edge of *XlSox-2*-expressing anterior neural plate (Fig. 1 C,E and D,F). Consequently, *ADAM13*-expressing area was located outside of *Xerl*-positive area (Fig. 1 A,E and B,F, right). Thus, neural crest-specific *ADAM13* expression occurred complementary to the cephalic expression of *Xerl* and *XlSox-2* (Fig. 1F, right, arrowhead). These data showed that *Xerl* expression was specific in CNS before and after neural folding.

Morphological changes in *Xerl*-overexpressing embryos

For gain-of-function analysis, we performed microinjection of synthesized mRNA including full ORF of *Xerl* into one blastomere of 4-cell stage embryo. Green Fluorescent Protein (GFP) was used as a lineage tracer of injected blastomeres (Fig. 2A). At the stage of anterior neural plate closure, *GFP* mRNA-injected embryos indicated normal neurulation at all doses tested (Fig. 2B). However, *Xerl* mRNA-injected embryos showed incomplete neural closure (Fig. 2 C,D, arrow). About 40% embryos indicated the same phenotype (Table 1). *Xerl* mRNA was effective in the dorsal side, but the injection of *Xerl* into the ventral side did not lead any morphological changes (data not shown).

To know how *Xerl* overexpression causes the abnormal neurulation, we performed histological examination of *Xerl*-injected embryos. During early stages of neuru-

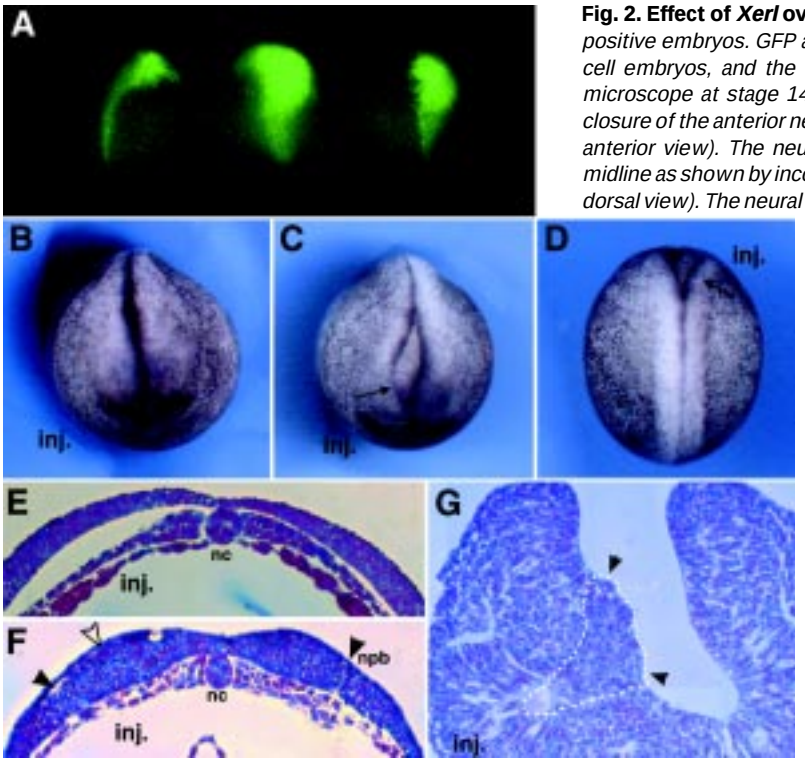

Fig. 2. Effect of *Xerl* overexpression on anterior neural folding. (A) *Selection of GFP-positive embryos. GFP and* Xerl *mRNA were co-injected into one dorsal blastomere of 4-cell embryos, and the GFP-positive injected side was recognized under fluorescence microscope at stage 14/15.* **(B)** *GFP mRNA-injected control (st.20, anterior view). The closure of the anterior neural plate was completed.* **(C)** Xerl *mRNA-injected embryo (st.20, anterior view). The neural fold in the injected side shifted to the distal-posterior from midline as shown by incomplete neural closure (arrow).* **(D)** *The same embryo of (C) (st.20, dorsal view). The neural fold in the injected side was smaller than the counter side (arrow).* **(E)** *Cross-section of the anterior neural plate of the* Xerl*-injected embryo (st.15). There was no significant change in the injected side (left).* **(F)** *The future mid-hindbrain level section of the anterior neural plate of the* Xerl*-injected embryo (st.17). Arrowheads indicate the neural plate boundary. Lateral shift of the neural plate boundary occurred in the injected side.* **(G)** *Cross-section of the future midbrain of the* Xerl*-injected embryo (st.20). Abnormal cell mass was observed in the injected side (broken-lined circle and arrowheads). inj, injected side; npb, neural plate boundary; nc, notochord.*

lation, *Xerl*-injected embryos showed no histological change at the anterior neural plate (Fig. 2E). However, in neurulating embryos at stage 17, the neural plate boundary of the injected side became obscure and its position shifted more laterally than that of the control side (Fig. 2F, arrowheads). At the end of neural folding, abnormal cell mass was observed in the injected side (Fig. 2G, arrowheads). The cell mass consisted of irregular-shaped cells, which was clearly different from columnar neuroepithelial cells (Fig. 2G, broken-lined circle).

Xerl overexpression causes neural plate expansion

To know what kind of tissues is induced within the *Xerl*-injected side, we examined gene expression of tissue-specific markers using whole mount *in situ* hybridization at the early neurula stage. First, we examined neural plate marker, *XlSox-2*. In control embryos, *XlSox-2* expression was symmetrical at neural plate (Fig. 3A, left, 100%). However, *Xerl*-injected embryos showed wider expression of *XlSox-2* at the injected side than the control side (Fig. 3A, right, 68%). NCAM-positive area at the anterior neural plate expanded and slightly at the trunk neural plate, which directly demonstrated the neural plate expansion (Fig. 3C, right, arrowhead, 42%). Gene expression of epidermal marker, *XK81A1* showed posterior shift in the anterior region (Fig. 3B, right, arrowheads, 45%). These results indicate that neural plate expansion occurs in the *Xerl*-injected embryo. *Xerl* overexpression did not diminish *ADAM13*-positive neural crest cells, but caused posterior shift of the neural crest (Fig. 3D, right, 44%). To confirm this phenotype, we examined another neural crest marker, *Xslug* expression. *Xslug* expression was also shifted to outside of expanded neural plate (Fig. 3E, right, 53%). As described above, *Xerl* overexpression caused the incomplete folding of the anterior neural tube and the formation of abnormal cell mass (Fig. 2G). However, *XlSox-2* expression was seen even in the deformed neural area (Fig. 3G, right, arrow). Histological observation revealed that *XlSox-2* expression occurred in *Xerl*-induced abnormal cell mass (Fig. 3H, opened arrowhead). In *Xerl*-injected embryo, posterior shift of neural crest was still recognized at the stage of late neurula, and the exclusion of *ADAM13* expression was seen in the abnormal cell mass (Fig. 3F, right, arrow).

TABLE 1

EFFECT OF *XERL* OVEREXPRESSION

	Injected samples			
Phenotype	*Xerl* 2 ng		*GFP* 2 ng	
Incomplete neural closure	49	(39%)	1	(4%)
Normal neurogenesis	77	(61%)	27	(96%)
Total	126	(100%)	28	(100%)

Synthesized mRNAs of *Xerl* or *GFP* were injected into one dorsal blastomere of 4-cell embryos. Effect of Xerl on neurogenesis was examined at stage 20. The characteristic of phenotype is the opened anterior neural plate and the small neural fold, which were categorized into one group as incomplete neural closure.

Antisense *Xerl* injection up-regulates gene expression in the neural crest

Xerl overexpression caused neural plate expansion. Then, in order to examine whether an inhibition of Xerl function causes defective formation of neural plate, we performed antisense *Xerl* injection. As compared with the normal embryo, the antisense *Xerl*-injected embryo showed neural fold overgrowth in the injected side (Fig. 4 A-C). The histological analysis revealed that overproduction of the neural fold occurred widely in the dorsal ectoderm beyond the neural plate boundary (Fig. 4D, opened arrowhead).

In order to determine the type of cells overproduced in the antisense *Xerl*-injected side, we examined gene expression of *XlSox-2* and *ADAM13* in injected embryos. Whole mount *in situ* hybridization showed that antisense *Xerl* injection caused a reduction of *XlSox-2* expression (Fig. 4E, 56%). In contrast, gene expression of neural crest marker, *ADAM13* was clearly en-

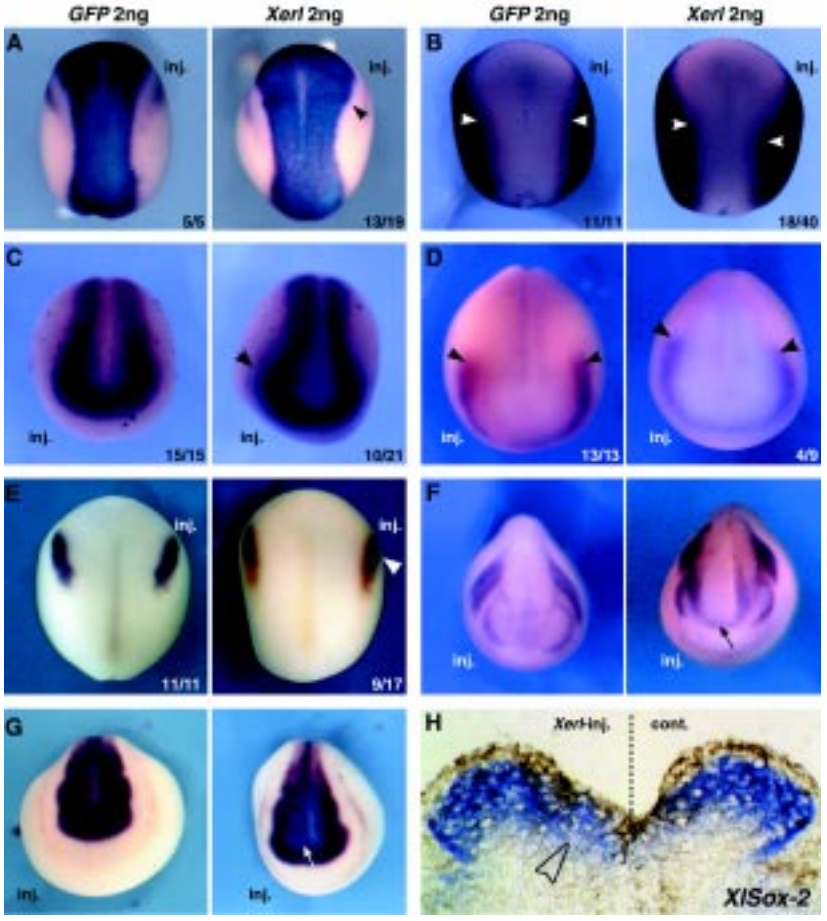

Fig. 3. Gene expression in the *Xerl*-injected embryo. *Synthesized mRNAs of GFP or Xerl were injected into one dorsal blastomere of 4-cell stage embryos, and neural marker gene expression was examined by whole mount* in situ *hybridization at the stage 15 (A-E) or 20 (F and G).* **(A)** XlSox-2 *expression showing the neural plate area (dorsal view). Arrowhead indicates expansion of the* XlSox-2*-positive area in the* Xerl*-injected side.* **(B)** XK81A1 *expression showing the epidermal area (dorsal view). Arrowheads indicate the posterior end of the head neural plate.* **(C)** NCAM *expression showing the neural plate area (anterior view). Arrowhead indicates expansion of* NCAM *expression.* **(D)** ADAM13 *expression showing future cephalic neural crest cells (anterior view). Arrowheads indicate the posterior ends of neural crest cells.* **(E)** Xslug *expression showing neural crest cells (dorsal view). Arrowhead indicates the outer and posterior shift of neural crest cells in the* Xerl *injected side.* **(F)** ADAM13 *expression showing cephalic neural crest cells.* ADAM13 *expression was not seen at the anterior disclosure.* **(G)** XlSox-2 *expression (st. 20, anterior view).* XlSox-2 *expression is seen in the* Xerl*-injected area (arrow).* **(H)** *Cross section at the midbrain level. The dorsal midline is indicated with the broken line. The opened arrowhead shows* XlSox-2 *expression in the abnormal tissues in the* Xerl*-injected side. Numbers in each picture indicate the frequency of the corresponding phenotype.*

hanced in the injected side (Fig. 4F arrowheads, 70%). As compared with the uninjected side, inner shift of *ADAM13*-positive area was observed in the antisense *Xerl*-injected side (Fig. 4F, arrows). Also, *Xslug* expression was increased in the antisense Xerl injected side (Fig. 4F, right, 71%). In order to test whether or not the effect of Xerl on neurogenesis is mediated by chordin, gene expression of chordin was examined in the antisense *Xerl*-injected embryo. However, as shown in Figure 4G, antisense *Xerl* did not change the expression pattern of *chordin.* In order to confirm the inhibitory effect of antisense RNA, double stranded RNA was used as RNAi for suppressing Xerl function. As shown in Table 2, the down regulation of *XlSox-2* expression and the inner shift of *ADAM13*-expressing area were recognized in the Xerl RNAi-injected embryos.

Xerl is essential for neural plate/ neural crest boundary formation

Foregoing results present a possibility that Xerl promotes neural plate formation. However, overexpression of *Xerl* produced abnormal population having neural plate-identity. To examine a function of Xerl in neurogenesis in detail, we considered conjugated animal cap assay (Fig. 5A). In this system, the chordin-injected wild animal cap is the neurogenic center, and this tissue induces gene expression in the conjugated albino animal cap that has been previously injected with test samples. As dispersion of chordin extends from the original cap to the neighboring one (LaBonne and Bronner-Fraser, 1998), the effect of test samples on chordin-induced gene expression can be examined in the albino side. Consequently in this assay system, stable inducing signal can be obtained from the dose-sensitive chordin, since the inducing tissue is segregated from the reacting tissue. One nano gram of chordin was injected into both blastomeres at 2-cell stage, which can induce neurogenesis in the isolated animal cap. In *chordin*/control conjugates, *Xerl* was induced in both caps by chordin (Fig. 5B). *Xerl* induction by chordin was also confirmed with RT-PCR analysis of the single *chordin*-injected animal cap (data not shown). Gene expression of *XlSox-2* was induced in the chordin-injected animal cap and even in the neighboring area of control cap (Fig. 5C). In *chordin/Xerl* conjugates, gene expression of *XlSox-2* was observed in whole area of the *Xerl*-injected albino cap (Fig. 5D). In contrast, *XlSox-2* expression was not detected in antisense *Xerl*-injected albino cap of chordin/antisense *Xerl* conjugates (Fig. 5E). Since *XlSox-2* did not induce *Xerl,* and *Xerl* could not induce the sufficient expression of *XlSox-2* (Fig. 5 F,G), these results suggest that the expression of *XlSox-2* requires both chordin and Xerl.

In order to know Xerl function in the neural crest formation, we examined *ADAM13* expression in this conjugated animal cap assay. *Chordin*/control and *chordin/Xerl* conjugates showed no expression of *ADAM13* in both caps (Fig. 5 H,I). However, in *chordin* /antisense *Xerl* conjugates, *ADAM13* expression was induced in the antisense *Xerl*-injected side (Fig. 5J). These data indicated that Xerl inhibition under the neural induction by chordin could induce the neural crest formation. This result was confirmed by co-injection of *chordin* and antisense *Xerl* in animal cap. Chordin could not induce gene expression of *ADAM13*, whereas co-injection of chordin and antisense *Xerl* induced ectopic expression of *ADAM13* (Fig. 5 K,L).

Xerl lacking the LNS domain acts as a dominant negative form

As shown above, antisense RNA injection and RNAi experiment caused the reduction in the neural plate formation or neural fold overgrowth. However, there are many arguments for the

antisense RNA and RNAi methods. To resolve this problem, truncated forms of Xerl were used for the functional analysis. Xerl has two major domains; one is calcium binding EGF-like repeats (cbELRs) and the other is LNS domain. Xerl lacking cbELR3-10 and EGF11 is named as Xerl ΔE. Also Xerl lacking LNS domain is named as Xerl ΔL (see the scheme of Fig. 6A). When truncated-RNAs were injected into one blastomere at 2-cell stage, *Xerl ΔE* caused the anterior disclosure of the neural plate (Fig. 6B, opened arrowhead). This result is the same as that in the overexpression of *Xerl* (Fig. 2 C,G). *Xerl ΔL* did not cause any anterior disclosure (data not shown). In contrast to the injection with *Xerl ΔE,* the injection with *Xerl ΔL* caused the inner shift of neural fold (Fig. 6C, opened arrowhead), and the overproduction of the neural fold (Fig. 6C, white arrowhead). These data suggest that Xerl ΔL acts as a dominant negative form of Xerl. To confirm this idea, we examined gene expression in the embryos injected with truncated RNAs. *Xerl ΔE* caused the distal-posterior shift of *ADAM13* expression (Fig. 6D, arrowhead, 65 %). In contrast, *Xerl ΔL* caused the anterior shift and the overgrowth of *ADAM13*-positive neural fold cells (Fig. 6E, arrowhead, 49 %). These data indicate that Xerl ΔL acts as a dominant negative form, which causes the same phenotype as the antisense RNA and RNAi-injected embryos.

Discussion

Xerl excludes neural crest cells

In this study, antisense *Xerl* injection showed the differentiation of large amount of neural crest cells. Furthermore, antisense *Xerl* injection induced gene expression of *ADAM13* under existence of chordin. Judging from these results, Xerl seems to suppress the neural crest formation. However, overexpression of *Xerl* did not diminish the neural crest cells, but caused the outer shift of their distribution. In contrast, the inner shift of *ADAM13* expression was observed in the antisense *Xerl*-injected embryo. These results indicate that one of the most important roles of Xerl is to exclude neural crest differentiation from the presumptive neural plate region. If this is the case, Xerl must have a molecular mechanism to suppress

TABLE 2

EFFECTS OF XERL INHIBITION ON NEURAL PLATE/NEURAL CREST FORMATION

				positive effects		negative effects	
probes	sample	n	normal	increased	inner shift	decreased	outer shift
XlSox-2	*GFP*	5	100%	0%	-	0%	-
	asXerl	18	44%	0%	-	56%	-
	Xerl RNAi	10	30%	0%	-	70%	-
ADAM13	*GFP*	13	100%	-	0%	-	0%
	asXerl	10	30%	-	70%	-	0%
	Xerl RNAi	5	20%	-	80%	-	0%
Xslug	*GFP*	15	100%	-	0%	-	0%
	asXerl	7	29%	-	71%	-	0%
	Xerl RNAi	NT	-	-	-	-	-

Whole mount *in situ* hybridization analyses were performed on early neurulas (st. 15). One dorsal blastomere of 4 cell embryos was injected. The effect of injected samples was judged by comparison with non-injected control sides. The embryo showing symmetrical expression of each probe was indicated as normal. Effects of the injection were evaluated by quantitative (*XlSox-2*) or local shift of gene expression (*ADAM13, Xslug*). NT, not tested.

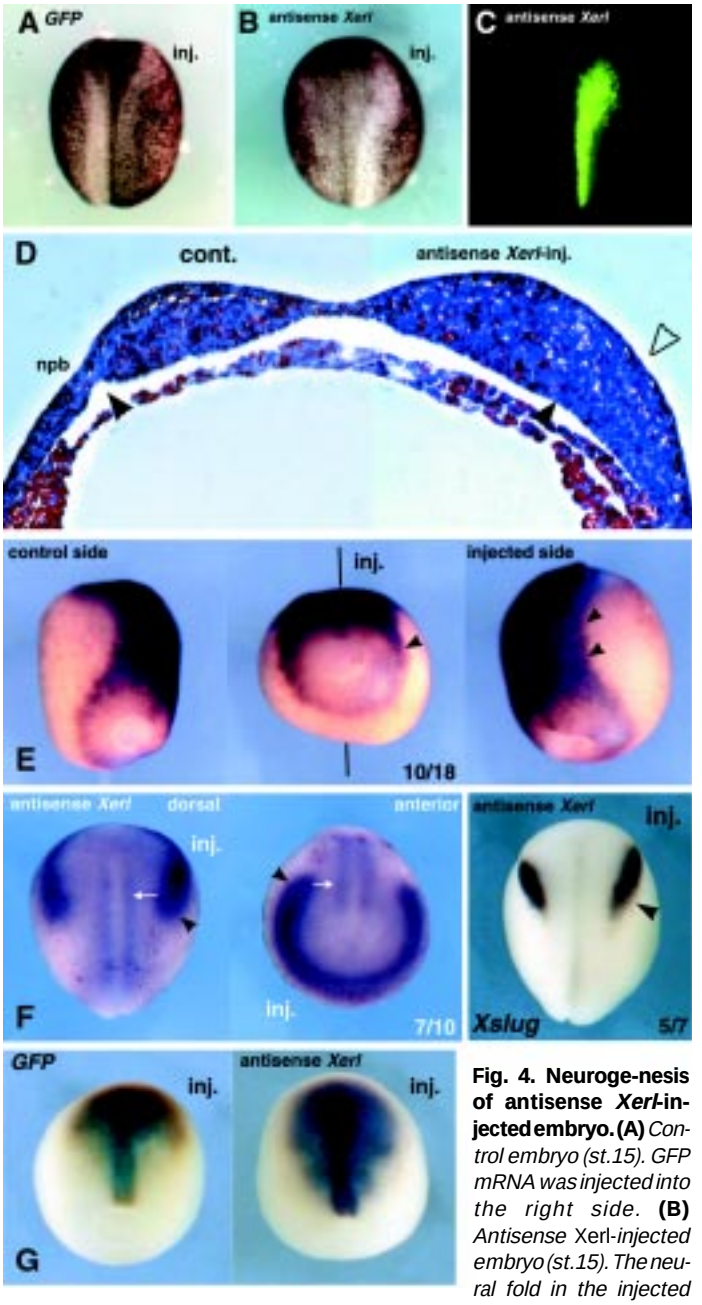

Fig. 4. Neuroge-nesis of antisense *Xerl*-injected embryo. (A) *Control embryo (st.15). GFP mRNA was injected into the right side.* **(B)** *Antisense* Xerl*-injected embryo (st.15). The neural fold in the injected side was larger than that of the control side.* **(C)** *GFP fluorescence showing the injected side of B.* **(D)** *Cross section on the anterior neural plate of (B). The injection was performed to the right. Arrowheads indicate the neural plate/neural crest boundary. The opened arrowhead at the injected side indicates the overproduction of the neural fold cells.* **(E)** XlSox-2 *expression (st.12). Lateral and marginal expression of* XlSox-2 *in the injected side (right) was fainter than that in the control side.* **(F)** *(Left)* ADAM13 *expression in the antisense* Xerl*-injected embryo (st.15). Arrowheads indicate enhanced expression of* ADAM13 *in the injected side. Inner shift of the* ADAM13*-expressing region (arrows) shows the reduction of the neural plate region. (Right)* Xslug *expression in the antisense* Xerl *injected embryo (st.15). Arrowheads indicate an increase and inner shift of expression.* **(G)** Chordin *expression (st.12). Normal* chordin *expression in the axial mesoderm was observed in control and in the antisense* Xerl*-injected embryos. Numbers on the picture indicate the frequency of the corresponding phenotype. inj, injected side; cont, control side; npb, neural plate boundary.*

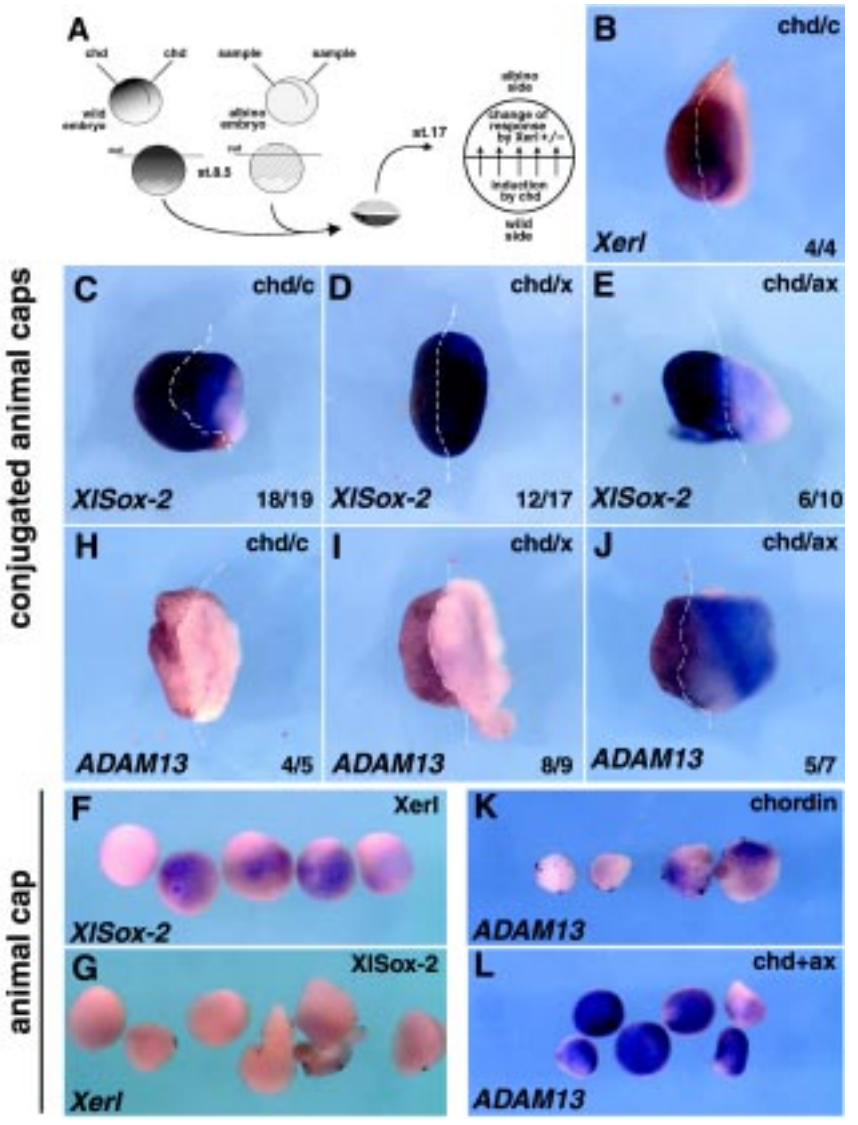

Fig. 5. Conjugated animal cap assay for testing chordin-Xerl interaction. (A) *Schematic diagram illustrating the conjugated animal cap assay. Conjugates were made by combining animal cap from* chordin*-injected embryos (wild) and animal cap from experimental embryos injected with various test samples (albino) at stage 8.5. Gene expression was examined by whole mount hybridization at stage 17. (B-L) The upper-right corner indicates the injected sample or combination of conjugates. The lower-left corner indicates the probe used for gene expression analysis. The lower-right corner indicates the count of conjugates expressing the same phenotype.* **(B)** Xerl *expression in* chordin*/control conjugates.* Xerl *expression was seen at the center of the conjugate.* **(C)** XlSox-2 *expression in chordin/control conjugates.* XlSox-2 *expression was seen near the border.* **(D)** XlSox-2 *expression in* chordin/Xerl *conjugates.* XlSox-2 *expression was seen in the whole conjugate.* **(E)** XlSox-2 *expression in* chordin*/antisense* Xerl *conjugates.* XlSox-2 *expression was not seen in the antisense* Xerl*-injected side.* **(F)** XlSox-2 *expression in* Xerl*-injected animal caps. Sufficient* XlSox-2 *expression was not detected, although a weak signal was seen in some specimens.* **(G)** Xerl *expression in* XlSox-2*-injected animal caps. No expression was observed.* **(H)** ADAM13 *expression in* chordin*/control conjugates. No expression was observed.* **(I)** ADAM13 *expression in* chordin/Xerl *conjugates. No expression was observed.* **(J)** ADAM13 *expression in* chordin*/antisense* Xerl *conjugates. The expression was clearly recognized in the antisense* Xerl*-injected side.* **(K)** ADAM13 *expression in* chordin*-injected animal caps. No expression was observed.* **(L)** ADAM13 *expression in animal caps co-injected with* chordin *and antisense* Xerl. *Ectopic* ADAM13 *expression was induced.*

the PNS-inducible circumstance. In recent reports, several inducing signals like Wnts or FGFs are involved in the expression of *Xslug* for neural crest differentiation (Mayor *et al*., 1997, LaBonne and Bronner-Fraser, 1998). Xerl maybe act as an antagonist of the neural crest-inducing signal, since Xerl has several characteristics as a signaling molecule. One of characteristic domains is calcium binding EGF-like repeat (cbELR). Notch ICD and its downstream gene, *Xash3* cause anterior disclosure, and are also involved in neural plate and crest development (Turner and Weintraub, 1994). The signal between Notch and Delta is directly transmitted by cbELRs (Rebay and Fleming, 1991). However, the truncation experiment revealed that Xerl does not need cbELR domains for its function. This is similar to the case of WIF-1. The Wnt-inhibiting molecule has ELR and WIF domain and does not need ELR for Wnt inhibition (Hsieh *et al*., 1999). They guess that ELR may be required for binding with the extracellular matrix. In contrast, the present study showed that functional domain of Xerl is the LNS domain. There is a report suggesting that LNS domain is also molecular interacting domain on the cell surface (Missler and Südhof, 1998). However, there has been no report connecting LNS molecule with Wnt or FGF signaling. Slit is an axon guidance molecule, which have leucine-rich repeats, ELRs and LNS domain. Also in this case, each domain's function is still unknown. Recent report showed that LNS domain has a new molecular site interacting with sugars, steroid and proteins (Rudenko *et al.*, 2001). Therefore, Xerl may act as a signaling molecule mediating unknown pathway. Thus, further study is necessary for examining whether Xerl functions as one of antagonistic factors against PNS-inducing signals.

Xerl promotes neural plate formation

In this study, *Xerl* overexpression caused *XlSox-2* positive-neural plate expansion and the opened anterior neural plate, which was due to produce *XlSox-2*-positive neuroepithelial cell mass. *Sox-2* alone is not sufficient for neural induction, but enhances the responsiveness of ectoderm to the neuralizing FGF signals (Mizuseki *et al*., 1998). Also, in chick embryos, *Sox-2* function is related to competence for neural induction (Streit A. *et al*., 1997). Therefore, one role of Xerl may be to provide the competence for CNS-inducible signals to the neuroepithelium through the promotion of *XlSox-2* expression. The next question is how Xerl promotes *XlSox-2* expression. One

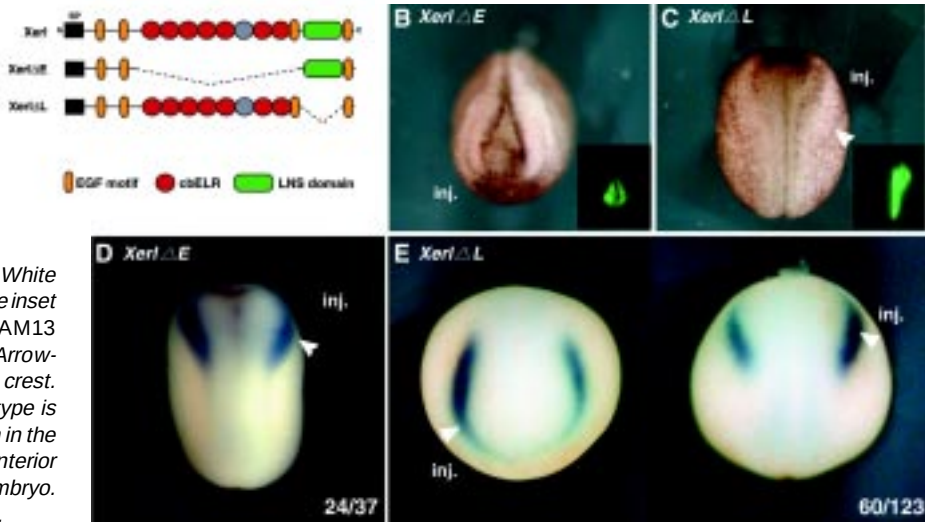

Fig. 6. Truncated Xerl overexpression. **(A)** *Scheme illustrating truncated constructs.* **(B)** *Anterior view of the* Xerl ΔE-*injected embryo (st. 20). Opened arrowhead indicates extra epithelial tissues within the anterior disclosure. The inset is a fluorescent view of the same embryo.* **(C)** *Dorsal view of the* Xerl ΔL-*injected embryo (st.17). Opened arrowhead indicates the inner shift of the end of the anterior neural fold. White arrowhead indicates the overgrowth of neural fold. The inset is a fluorescent view of the same embryo.* **(D)** ADAM13 *expression in the* Xerl ΔE-*injected embryo (st.15). Arrowhead indicates the distal-posterior shift of the neural crest. The number of embryos showing the same phenotype is indicated at the right corner.* **(E)** ADAM13 *expression in the* Xerl ΔL-*injected embryo (st.15). The left panel is the anterior view. The right panel is the dorsal view of the same embryo. Arrowhead indicates the overgrowth of neural crest.*

possible mechanism of *XlSox-2* promotion is that Xerl may interact with chordin directly in the extracellular space. Blitz *et al.* (2000) showed that chordin-processing by BMP-1 or Xolloid makes the different activity of BMPR. However, *BMP-1* expression is ubiquitous, and *Xolloid* expression is seen in the lateral neural plate in the early neurula embryo (Goodman *et al.*, 1998). If Xerl protects chordin from BMP-1 or Xolloid in the neural plate area, distribution of active chordin could be refined by expression pattern of *Xerl* in CNS.

Consequently, Xerl must play an important role for neural plate/neural crest boundary formation.

Xerl expression and regulation

Present study showed that chordin induces gene expression of *Xerl*. In the early neurula stage, *Xerl* expression was similar to chordin expression (Sasai *et al.*, 1994). However, at the later stage, *chordin* expression is restricted to only forebrain and chordoneural hinge (Sasai *et al*., 1994). Whereas, *Xerl* expression is seen at the ventricular zone of diencephalon and midbrain at the early tadpole stage (Kuriyama *et al*., 2000). Thus, the expression pattern of *Xerl* was wider than that of *chordin* expression. Presumably, *Xerl*-inducible factor is not only *chordin* at the later stage.

Materials and Methods

Embryos

Unfertilized eggs were obtained by intracoelomic injection with gonadoropic hormone (Gestron; Denka Seiyaku, Tokyo, Japan) into *Xenopus* adult females and artificial fertilization *in vitro* were performed as described by Asashima *et al*. (1990). Fertilized eggs were treated with 1 % thioglycoate (pH 10) and cultivated in 0.1x Modified Barth's Solution (MBS) at 20°C (Moon and Christian, 1989). Developmental stage was according to Nieuwkoop and Faber (1967).

Expression construct and microinjection

A full-frame *Xerl* expression plasmid, termed pCS2+/*Xerl*, was constructed in the expression vector pCS2+. The fragment was digested with *Avi*II and *Xho*I and inserted into the vector. The capped pCS2+/Xerl, pCS2+/chordin and pCS2+/myc-tagged GFP (mtUGP) mRNAs were transcribed using SP6 RNA polymerase as described in the procedure of mCAP RNA Capping Kit (STRATAGENE). RNAs were injected in a volume of 5 nl at a concentration of 50–400 pg/nl into one single blastomere of embryos at stage 2 or 3. Microinjections were done in 0.1 x MBS, 2.5% Ficoll 400 (Amersham Pharmacia Biotech). After gastrulation, injected embryos were transferred to 0.1 x MBS for further incubation (Moon and Christian, 1989). In all experiments, pCS2+/mtUGP mRNA was used as control.

Antisense RNA and RNAi construct

Antisense RNA injection have been used to inhibit the function of target gene in *Xenopus* (Steinbeisser *et al*., 1995, Carl *et al*., 1999). Antisense RNA was synthesized from the first 1.6 kb of 2.9 kb cDNA including 5' flanking region and the initiating codon. *Xenopus* RNAi was reported by Nakano *et al.* (2000). RNAi was synthesized from full-length form of Xerl, non-capped antisense RNA and sense RNA were determined by gel-electrophoresis and spectrophotometry. Then, these RNAs were denatured and annealed, and size-up of this double stranded RNA was checked by electrophoresis as described by Li *et al*. (2000).

Truncated constructs

Truncated constructs of *Xerl* were made by PCR and replacement of digested cDNA fragment. *Xerl ΔE* was lacking the position of 323 to 1799 bp fragment. *Xerl ΔL* was lacking the position of 1630 to 2288 bp fragment.

Histological analysis

All injected embryos were selected under fluorescence microscope. For histological analysis of injected embryos, GFP-positive embryos were fixed with 1 x MEMFA for overnight. Embryos were embedded in metacryl resign, marked on the injected side and sectioned with ultra microtorme. Staining was performed by conventional azofuchsin-anilin blue method as described by Hausen (1988).

In situ hybridization analysis

Whole mount *in situ* hybridization was performed as described previously (Kuriyama *et al*., 2000). Minor modification was done in a coloring reaction using BM purple (Roche Diagnostics) at 4°C for overnight. For histological examination of stained embryos, embryos were fixed with 1 x MEMFA for overnight, embedded in metacryl resign, and sectioned with 2-8-μm thickness. Bleaching of wild type embryos for *in situ* hybridization was performed by using 10 % H_2O_2 in PBS only for samples shown in Fig. 6 D,E.

Conjugated animal cap assay

Animal caps from wild and albino embryos were used to distinguish the donor from the recipient. Wild embryos were injected at the stage 2 with 1 ng *chordin* mRNA, and albino embryos with 2 ng *Xerl* or antisense *Xerl* mRNA. These injected embryos were incubated in 0.1 x MBS to stage 8.5, when they were transferred into 1 x MBS, their vitelline membranes were removed with watchmaker's forceps, and animal caps were quarried with tungsten needle.

Wild and albino animal cap were immediately conjugated with each other, washed with 1 x MBS and cultured until stage 17. Except above modification, all procedures of conjugate assay were performed as described by LaBonne and Bronner-Fraser (1998).

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