Int. J. Dev. Biol. 57: 779-786 (2013)
doi: 10.1387/ijdb.130161tm

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Xnr3 affects brain patterning via cell migration in the neural-epidermal tissue boundary during early *Xenopus* embryogenesis

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ABSTRACT Neural induction and anteroposterior neural patterning occur simultaneously during *Xenopus* gastrulation by the inhibition of BMP and Wnt signaling, respectively. However, other processes might be necessary for determining the neural-epidermal boundary. *Xenopus nodal-related-3* (*Xnr3*) is expressed in dorsal blastula and plays a role in neural formation. In this study, we analyzed how *Xnr3* affects neural patterning to identify novel mechanisms of neural-epidermal-boundary determination. *In situ* hybridization revealed that ventro-animal injection with *Xnr3* shifted the lateral *krox20* expression domain anteriorly and reduced *Otx2* expression. The mature region of *Xnr3* is necessary for these effects to occur, and the pro-region accelerated them. Phalloidin labeling revealed that cells around the neural-epidermal boundary lost their slender shape following *Xnr3* injection. Moreover, we analyzed the cell migration of ectodermal cells and found specific *Xnr3*-induced effects at the neural-epidermal boundary. These findings together suggested that *Xnr3* affects anterior ectoderm migration around the neural-epidermal boundary to induce a specific neural pattern abnormality. Change of the shape of surrounding ectodermal cells and the specific migratory pattern might therefore reflect the novel mechanism of neural-epidermal boundary.

KEY WORDS: *Xenopus, Xnr3, neural-epidermal boundary, ectoderm, neural patterning, cell migration*

Introduction

The neural tissue of *Xenopus laevis* acquires its complex structure through complicated processes. Neural induction is thought to occur following migration of the dorsal mesoderm, known as the Spemann organizer, and subsequent contact with the presumptive neuroectoderm during gastrulation. Genes that encode neuralizing factors such as *noggin*, *chordin*, and *follistatin* are expressed in the dorsal mesoderm (Smith and Harland, 1992; Lamb *et al*., 1993; Smith *et al*., 1993; Sasai *et al*., 1994; Hemmati-Brivanlou *et al*., 1994), and the encoded proteins induce neural tissue formation by antagonizing bone morphogenetic protein (BMP) signaling in the neuroectoderm (Sasai *et al*., 1994; Zimmerman *et al*., 1996; Hemmati-Brivanlou *et al*., 1994).

The detailed patterning of the neural region along the anteroposterior (A-P) axis also occurs during gastrulation. The anterior mesendoderm (also called the head organizer) induces anterior structures, whereas the chordamesoderm (called the trunk-tail organizer) induces development of the spinal cord. Previous studies have shown that canonical Wnt signaling plays an important role in such A-P neural patterning. Indeed, inhibition of both BMP signaling and canonical Wnt signaling is necessary and sufficient to induce the head (Glinka *et al.,* 1997). Anterior mesendoderm secretes *cerberus*, *dickkopf-1*, and *frzb,* all of which antagonize Wnt and promote anteriorization (Bouwmeester *et al*., 1996; Glinka *et al*., 1998; Leyns *et al*., 1997; Wang *et al*., 1997, Piccolo *et al*., 1999).

Neural patterning includes induction of the neural crest and anterior cranial placode between the epidermis and neural plate,

Abbreviations used in this paper: BMP, bone morphogenetic protein; DAP, dorsal animal pole; TGF-β: transforming growth factor-β; VAP, ventral animal pole; Xnr, Xenopus nodel-related.

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Accepted: 25 February 2013. *Final, author-corrected PDF published online:* 28 June 2013. *Edited by:* Makoto Asashima.

ISSN: Online 1696-3547, Print 0214-6282

and induction of these structures is required for appropriate levels of BMP, fibroblast growth factor (FGF), retinoic acid (RA), and Wnt signaling (Durston *et al.*, 1989; LaBonne and Bronner-Fraser, 1998; Villanueva *et al.*, 2002; Marchant *et al.*, 1998). However, recent studies indicated that formation of the neural region could require additional processes. For example, overexpression of BMP antagonists in competent chick epiblast does not always induce expression of neural marker genes and BMP protein had virtually no effect on neural plate development (Linker and Stern, 2004). In addition, early canonical Wnt signaling in *Xenopus* embryos induced expression of *chordin* and *noggin* at the BCNE (Blastula Chordin-Noggin Expressing) center, which contains presumptive neuroectoderm as well as the Spemann organizer (Kuroda *et al.*, 2004). Although mesoderm involution was prevented in dorsal marginal zone explants, the anterior neural tissue could be formed by BCNE center (Kuroda *et al.*, 2004). The above findings implicated an important role for the presumptive ectoderm in neural patterning. Moreover, *engrailed-2* (*en2*; expressed in midbrain-hindbrain boundary) and *xOtx2* (a head marker gene) are still expressed in Keller explants, suggesting that expression of several neural marker genes is independent of the contact between neuroectoderm and mesodermal tissue (Ruiz i Altaba 1992: Kuroda *et al.*, 2004).

Studies such as those mentioned suggested that neural patterning is determined by as yet undetermined mechanisms involved before gastrulation. We focused on the boundary between the dorsal side and the ventral side at the 4-cell stage, roughly corresponding to the epidermal-neural boundary of neurula. To investigate such a hypothesis, this study analyzed differences in gene expression of blastula animal pole region between the dorsal side (Dorsal Animal Pole (DAP)) and the ventral side (Ventral Animal Pole (VAP)). Using DNA microarray analysis, we found that > 100 genes were more intensely expressed in the DAP than in the VAP. One of these, *Xenopus nodal-related gene 3* (*Xnr3*), was expressed at a few hundreds-fold higher on the dorsal side than on the ventral side (data not shown).

Xnr3 is one of six nodal-related genes, which belong to the transforming growth factor-β (TGF-β) superfamily in *Xenopus* (Smith *et al.*, 1995; Jones *et al.*, 1995; Joseph and Melton, 1997; Takahashi *et al.*, 2000). However, *Xnr3* is distinct from other *Xnrs* in some aspects. For example, Xnr3 protein lacks the seventh cysteine that is highly conserved throughout the TGF-β superfamily (Ezal *et al.*, 2000). In addition, other Xnrs cooperate with VegT and Vg1 to induce the dorsal mesoderm, whereas Xnr3 does not (Jones *et al.*, 1995; Hansen *et al.*, 1997; Agius *et al.*, 2000; Hyde and Old, 2000). Xnr3 is also expressed in presumptive dorsal ectoderm at the blastula stage (Smith *et al.*, 1995), whereas other Xnrs are secreted at the Nieuwkoop center located in the dorsal vegetal region (Jones *et al.*, 1995; Joseph and Melton, 1997; Takahashi *et al.*, 2000).

Nodal ligands are produced as precursor proteins that consist of a pro-region and a mature region. Once precursor proteins form disulfide-linked homodimers, the pro-region is then cleaved from the ligand (Massagué 1990; Agius *et al.*, 2000). The nodal pro-protein is relatively stable, whereas the processed mature ligand is readily degraded after cellular internalization (Haramoto *et al.*, 2004). In the case of Xnr3, the mature ligand does not activate the Smad pathway, but does induce the expression of *Xenopus brachyury* (*Xbra*) through the mitogen-activated protein (MAP) kinase pathway (Yokota *et al.*, 2003). Inhibition of *Xnr3* thus interferes with gastrulation, and especially with convergent extension of

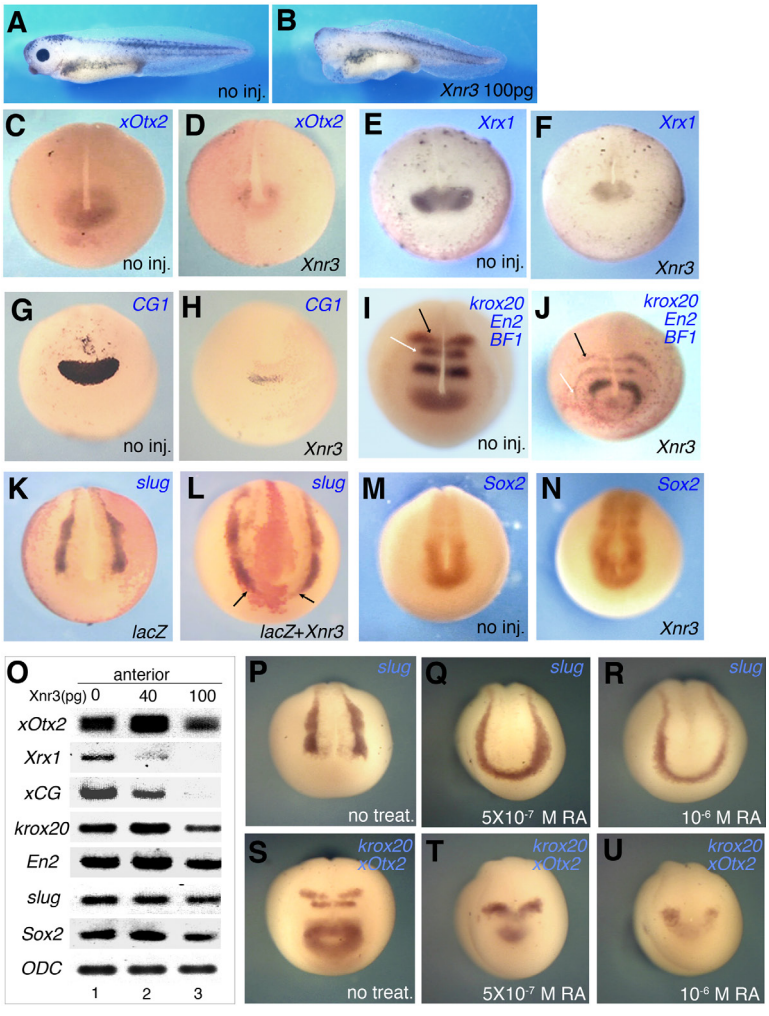


Fig. 1. Xnr3 injection induced an abnormal neural pattern in *Xenopus* embryos. (A,B) *Superficial phenotype of 2-day tadpole after Xnr3 injection.* **(A)** *An uninjected embryo.* **(B)** *An embryo injected with 100 pg of* Xnr3 *mRNA into the ventral animal pole (VAP) region at the 4-cell stage.* **(C-N)** *Whole-mount* in situ *hybridization (WISH) of stage-18 embryos. Normal embryos* **(C,E,G,I,K,M)** *and embryos injected with 40 pg of* Xnr3 *mRNA into the VAP region at the 4-cell stage* **(D,F,H,J,L,N)** *showing the spatial expression of* xOtx2 *(C, D),* Xrx1 *(E, F),* CG1 *(G, H),* krox20, En2, *and* xBF1 *(I, J),* slug *(K, L), and* Sox2 *(M, N). In (I,J),* krox20/En2 *expression extended laterally (black arrow) and curved anteriorly (white arrow). In* **(K,L)**, *200 pg of lacZ was also injected. Arrows in (L) show the anterior elongation of the* slug *expression domain.* **(O)** *Quantitative levels of brain marker genes measured by RT-PCR. Experiments were carried out with the anterior region dissected from stage-18 embryos. 0 pg (lane 1), 40 pg (lane 2), or 100 pg (lane 3) of* Xnr3 *mRNA were injected into the VAP of 4-cell embryos, and the expressions of* xOtx2 *(lane 1),* Xrx1 *(column 2),* xCG1 *(column 3),* krox20 *(column 4),* En2 *(column 5),* slug *(column 6),* Sox2 *(column 7) and* ODC *(column 8) were analyzed.* **(P-U)** *Expression patterns of neural markers in late neurula after treatment of the embryos with RA. Expression of slug (P-R),* krox20, *and* xOtx2 *(S-U). The embryos were treated with 5 x 10-7 M RA (Q,T) or 10-6 M RA (R,U).*

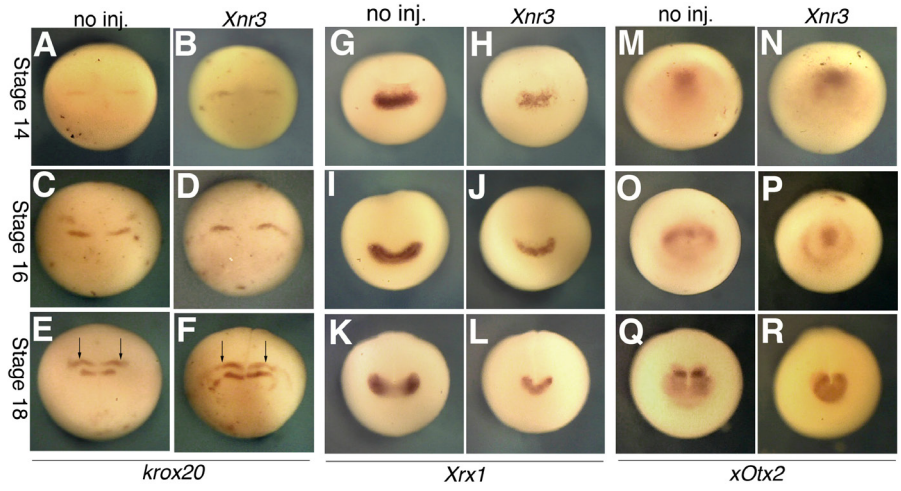


Fig. 2. Temporal changes in the specific neural pattern induced by Xnr3 injection. *100 pg of* Xnr3 *mRNA was injected into both ventro-animal hemispheres of 4-cell embryos (B,D,F,H,J,L,N,P,R). The expression of* krox20 **(A-F)**, Xrx1 **(G-L)**, *or* xOtx2 **(M-R)** *were observed at Stage 14 (A,B,G,H,M,N), Stage 16 (C,D,I,J,O,P) or Stage 18 (E,F,K,L,Q,R). The arrows in (E,F) indicate the neural-epidermal boundary.*

the dorsal mesoderm (Yokota *et al*., 2003). On the other hand, the pro-region of Xnr3 antagonizes BMP signaling by binding to BMPs after cleavage, resulting in induction of neural tissue (Haramoto *et al*., 2004).

In this study, we investigated the role of Xnr3 in neural patterning. Whole-mount *in situ* hybridization and RT-PCR analysis revealed that overexpression of Xnr3 induced a specific pattern of gene expression, especially at the border between the neuroectoderm and epidermis. The mature region of Xnr3 is sufficient for the specific patterning, but addition of the pro-region enhances the effect. A cell-lineage study showed that injection of *Xnr3* inhibited cell migration in the border region between the ventral and dorsal ectoderm. Normal embryos contained elongated cells in this region, and the direction of cell elongation followed the cell migration, whereas cells injected with *Xnr3* mRNA showed no elongation. These findings suggested that *Xnr3* exerts effects on cell movement that involve changes in cell shape in the border region between ventral and dorsal ectoderm, and in this way affects neural patterning.

Results

Overexpression of Xnr3 induced a characteristic pattern of neural gene expression

Previous studies reported that injection with *Xnr3* mRNA induces tail-like protrusions (Smith *et al*., 1995; Haramoto *et al*., 2004, 2007). When 100 pg of *Xnr3* was injected into the animal pole of 4-cell embryos in the present study, head defects were observed (Fig. 1A, B). Whole-mount *in situ* hybridization (WISH) to analyze the expressions of anterior neural marker genes revealed reduced regions of *xOtx2, Xrx1,* and *xCG* expressions in *Xnr3*-injected embryo at the late neurula stage (Fig. 1C-H). The lateral end of both *engrailed-2* (*En2*) and *krox20* expression domains curved anteriorly (Fig.1I, J, white arrow). Conversely, *Xnr3* minimally affected the marker gene expression patterns around the midline (Fig. 1I, J, black arrow). To examine the change in neural patterning induced by *Xnr3* injection in detail, we observed other marker genes. The neural crest marker, *slug,* was expressed at the neural-epidermal boundary in control embryos (Fig. 1K); however, *Xnr3* mRNA injection elongated the anterior end of the *slug* expression region (Fig. 1L). On the other hand, *Sox2* expression in the neural plate was laterally expanded by *Xnr3* mRNA injection, but the change was weak (Fig. 1M, N). We further examined quantitative level of the marker genes by RT-PCR analysis. The expression levels of *Xrx1,* and *CG1* were decreased, concordant with the WISH results (Fig. 1O, column 2, 3). *xOtx2* expression was decreased in embryos injected with 100 pg *Xnr3* (Fig. 1O, column 1). Conversely, the quantitative levels of *krox20*, *En2, slug*, and *Sox2* were not changed by *Xnr3* injection, suggesting that *Xnr3* did not inhibit the transcription of these latter genes (Fig. 1O, column 4-7).

Disappearance of the eyes and cement gland was also observed after posteriorization, thus we compared the neural pattern induced by *Xnr3* with that of posteriorized embryos induced by treatment with retinoic acid (RA). Slug expression extended anteriorly, and the region of *Xotx2* expression was reduced in RA-treated embryos, similar to that shown by *Xnr3*-injected embryos (Fig. 1P-R, S-U). On the other hand, 5 x 10^{-7} M RA neither elongated the *krox20* expression region nor shifted it anteriorly (Fig. 1T). In addition, embryos with either 5 x 10^{-7} M or 10^{-6}M of RA treatment showed fused expression regions of krox20 (Fig. 1T, U). These patterns were not observed in *Xnr3*-injected embryos (Fig. 1J). These findings corroborated evidence that the specific pattern of neural development induced by *Xnr3* is different from RT-induced posteriorization.

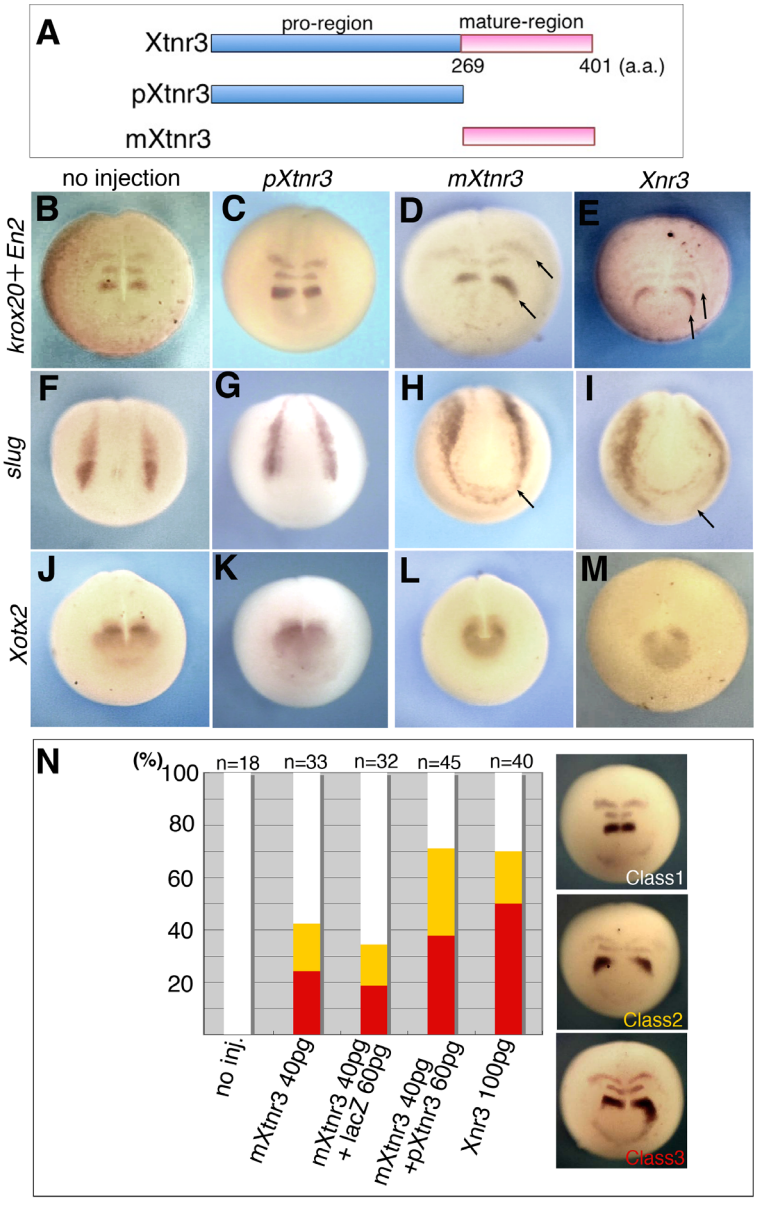
The severity of the Xnr3 effect on neural patterning increased during the mid-neurula stage

As described, late neurula-stage embryos showed clear and specific patterns of neural gene expression. To know when the specific neural patterns emerged, we examined the alteration of marker gene expressions across several stages of neurula. The *Krox20* pattern was hardly changed by stage 14 (Fig. 2A, B); however, by stage 16, the lateral domain of *krox20* gently curved toward the anterior region (Fig. 2C, D), and this curved pattern was obvious at stage 18 (Fig. 2E, F). The expression pattern of *Xrx1* was not largely altered, even though the expression level and area of expression was weak and small (Fig. 2G, H). At stage 16 and 18, *Xrx1*-positive regions were clearly shrunk (Fig 2I-L). Shrinkage of the *xOtx2* expression domain was also observed after stage 16 (Fig. 2O-R).

Both the mature region and pro-region of Xnr3 are important for specifying neural patterning

Like other members of the TGF-beta superfamily, Xnr3 functions after post-translational processing. The mature region of Xnr3 exerts the specific nodal protein function, whereas the pro-region shows BMP-antagonizing activity (Haramoto *et al*., 2004). Thus, we next sought to determine the *Xnr3* domain inducing the specific neural patterning. Herein, we used a *Xenopus tropicalis* homolog

of *Xnr3* (*Xtnr3*) that is functionally identical to *Xnr3* (Haramoto *et al.*, 2004). Injection of *pXtnr3* (Fig. 3A), encoding the pro-region of Xtnr3, into the VAP region hardly altered the expression patterns of *krox20*/*En2, slug,* and *xOtx2* (Fig. 3B-C, F-G, J-K). On the other hand, injection of the mature region of *Xtnr3* (*mXtnr3*; Fig. 3A) induced a bent pattern of *krox20* expression, similar to that in *Xnr3*-injected embryo (Fig. 3D, E). The expression regions of both *slug* and *Xotx2* were also smaller in *mXtnr3*-injected embryos, as in *Xnr3*-injected embryo (Fig. 3H-I and Fig. 3L-M). These results indicated that the mature region of *Xnr3* is essential for conferring the specific effects on neural patterning. However, the effects were only weak in *mXtnr3*-injected embryos, compared with *Xnr3* injections, prompting us to investigate the role of the *Xnr3* pro-region in pattern specification. Co-injection of *pXtnr3* and *mXtnr3* increased the severity of the specific effects on *krox20* and *En2* expression patterns to a level equivalent to that observed with full-length *Xnr3* (Fig. 3N). These results suggested that *pXtnr3* could enhance the specific function of *mXnr3* in neural patterning.



Xnr3 affects the shape of cells located between the ventral and dorsal ectoderm

Cell migrations are often accompanied with the changes in cell shape. During gastrulation, for instance, the cell shape of involute mesodermal zone becomes bipolar and slender as they move toward the midline during convergent extension. Thus we carried out phalloidin staining to examine whether abnormalities of cell shape was observed at the epidermal-neural boundary of *Xnr3*-injected embryo. The cells located at the epidermal-neural boundary were morphologically distinguished from dorsal cells and their shapes were elongated along the boundary, whereas the dorsal cells in the neural plate weakly elongated along the anterior-posterior axis (Fig. 4B-D). When *Xnr3* mRNA was injected, no slender cells were observed at the boundary (Fig. 4H-J). The lateral region of the boundary was similarly distinguished by specific shape of cells. Boundary cells in 7-8 rows also exhibited an elongated shape along the boundary (Fig. 4H-J). No slender cells were observed in the *Xnr3*-injected embryos (Fig. 4K-M).

Xnr3 influenced the migration of ectoderm cells at the neural plate boundary during the neurula stage

The observation of cell shape in *Xnr3* injected embryo showed that *Xnr3* overexpression inhibited bipolar cell formation at the neural-epidermal boundary. Based on the reported role of *Xnr3* in gastrulation movement in mesodermal tissue, we thought that the change in neural patterning elicited by *Xnr3* might happen via its effect on ectodermal cell migration. To analyze cell movements in *Xnr3*-injected embryo during neurulation, we carried out lineage-tracing assay with Alexa fluorescent dye. In normal embryos, Alexa488-positive cells located at the neural-epidermal boundary migrated posteriorly along the boundary (Fig. 5B, H left half). On the other hand, *Xnr3* injection reduced the migration of the Alexa488-positive cells (Fig. 5E, H right half). Dorso-anterior neuroectodermal cells also migrated along the boundary (Fig. 5C), whereas dorso-posterior cells migrated just posteriorly in normal embryo (Fig. 5D, I left half). *Xnr3* injected into the VAP region inhibited the migration of dorso-anterior cells (Figs. 5F, I right half), whereas the migration of dorso-posterior cells showed almost no difference between normal and *Xnr3*-injected embryos (Figs. 5G, I). However, cell migration was slightly disturbed in only the anterior end of neuroectoderm (Fig. 5I).

Fig. 3. The mature region of Xnr3 contributed to abnormal neural patterning. *(B-M) Spatial expression of neural marker genes was observed by WISH. 750 pg of* pXtnr3 *(C,G,K), 100 pg of* mXtnr3 *(D,H,L), or 40 pg of* Xnr3 *mRNA (E,I,M) was injected into the VAP region of 4-cell embryos.* pXtnr3 *and* mXtnr3 *indicate the pro-region and the mature-region, respectively, of* Xtnr3. *Embryos were examined with probes for* krox20 *and* En2 **(B-E)**, Xslug **(F-I)**, xOtx2 **(L-M)**. pXtnr3 *did not affect the expression patterns, whereas* mXtnr3 *injection induced patterns similar to those observed after injection of* Xnr3. *Arrow indicates the change in expression pattern.* **(N)** *Synergistic enhancement of the activity of* mXtnr3 *by* pXtnr3 *expression. Spatial expression of* En2/krox20 *at stage 18 was assessed by WISH, and the results are expressed in a bar graph. The severity of the effect neural is categorized into three classes: Class 1, Class 2 and Class 3 are represented by white, orange and red, respectively.*

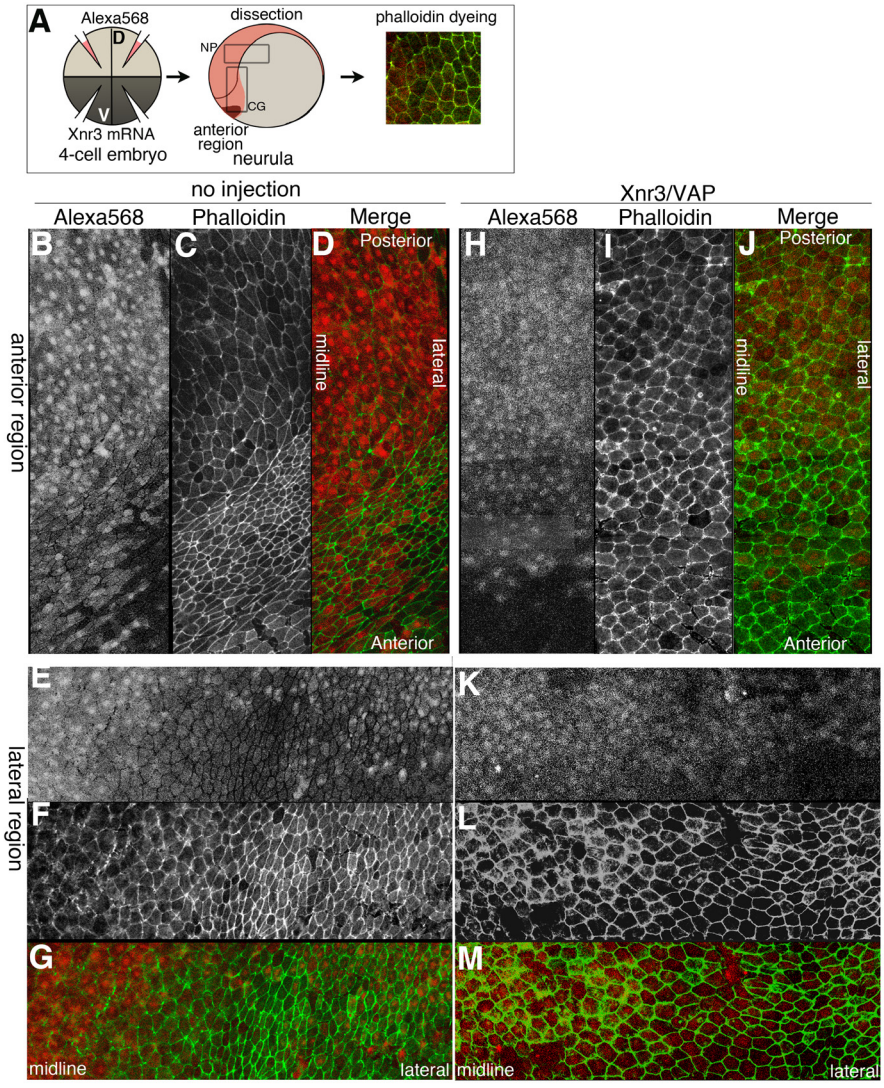


Fig. 4. Xnr3 injection specifically affected the shape of cells at the epidermal-neural boundary. (A) *The experimental procedure is illustrated in a schematic diagram. Alexa 568 and* Xnr3 *mRNA were injected into the DAP and the VAP region, respectively. At stage 18, the epidermal-neural boundary regions of the anterior end of neural plate and lateral neural plate were dissected and stained with phalloidin-Alexa488.* **(B-G)** *Double-fluorescent labeling of the anterior (B-D) or lateral (E-G) region of a normal embryo. The Alexa568 dextran staining pattern (B,E), the phalloidin staining pattern (C,F), and a merged image (D,G) are shown. The Alexa568-positive region approximately shows the presumptive neural plate.* **(H-M)** *Double-stained patterns in the anterior region (H-J) and lateral region (K-M) of an* Xnr3*-injected embryo. The Alexa568 dextran staining pattern (H, K), the phalloidin staining pattern (I, L), and a merged image (J, M) are shown.*

It is known that insufficient gastrulation can cause head defect with abnormal neural patterning. To eliminate this possibility for the effect by Xnr3 injection, we examined the distance between the anterior end of the neural groove and the anterior end of Alexa568-positive cells. This distance in the *Xnr3*-injected embryos was not different from that in normal embryos (Fig. 5J). Furthermore, sagittal sections of late neurula showed that neither the angle between the end of the dorsal ectoderm and dorsal lip nor the angle between the anterior end of the dorsal mesoderm and dorsal lip were altered by *Xnr3* injection (Fig. 5K). This result indicates that, in our experimental condition, *Xnr3* altered neither the extension of the dorsal ectoderm nor the final position reached by the dorsal mesoderm. Together, these results suggested that ectodermal cells at the neurula stage have region-specific motility and that *Xnr3* overexpression affects cell migration only in the ectodermal cells around the epidermal-neural boundary.

Discussion

In this study, we found that *Xnr3* overexpression caused specific changes in neural patterning during *Xenopus* embryogenesis. These changes were: (1) shrinkage of the anterior end of the neural plate, and (2) bending of the *krox20* pattern and anterior elongation of neural crest. As a reason for the shrinkage of the anterior end of the neural plate, posteriorization effect was thought. RA-treated embryos showed virtually no *krox20* expression (Fig. 1), while in *Xnr3*-injected embryos, *krox20* expression was still observed even though the spatial pattern was altered, suggesting that the effect of *Xnr3* was distinguishable from that of RA-induced posteriorization. Abnormal gastrulation could also be causally involved, and indeed, *Xnr3* affects gastrulation movements (Herrmann *et al.*, 1990; Schulte-Merker and Smith, 1995; Wilson *et al.*, 1995; Tada and Smith, 2000). However, our results showed the leading edge of the dorsal mesoderm arriving at the anterior end in *Xnr3*-injected embryos (Fig. 4), thus the specific neural pattern induced by *Xnr3* is likely to be independent of the gastrulation defect. As described in Fig. 5, migration of the anterior end of the dorsal ectoderm was also disrupted by *Xnr3* injection, and this shrinkage could be related to the anterior migratory defect.

The most characteristic change in neural patterning induced by *Xnr3* was lateral extension and anterior curvation of the *krox20* expression domain. We first speculated that such an alteration reflected a change of positional information in the anterior neuroectoderm. However, phalloidin staining revealed that *Xnr3* overexpression inhibited lengthening of cells along the boundary (Fig. 4). Furthermore, the cell tracing experiments suggested that the defect was caused by specific inhibition of cell migration at the neural-epidermal boundary. Thus, we propose that the loss of cell polarity could lead to cause the migration defect in the boundary cells, resulting in the bend of krox20 pattern.

The epidermal region adjacent to the neural plate is necessary for both neural plate extension and neural tube closure (Jacobson and Moury, 1995). Neural-epidermal boundary cells also contribute to the precise formation of the neural region, although the planar movements at the boundary have not been investigated in detail. Our study provides a novel model in which specific movements by boundary cells are important in neural pattern formation. Cell

adhesion may be involved in such specific movement changes. Indeed, *Xbra* inhibits cell migration by impairing adhesion between mesodermal cells and fibronectin *in vitro* (Kwan and Kirschner, 2003). Another possibility is that the specific cell movements at the neural-epidermal boundary depend on orientation of the ECM along the boundary, and further studies should be carried out to clarify the involvement of these factors.

It was previously shown that injection with morpholino antisenseoligo against Xnr3 (Xnr3MO) into dorsal equatorial region caused curvature and shortening of axis and loss of head structure (Yokota *et al.,* 2003). Though animal injection was not done, these results show that Xnr3 is, at least, required for proper head formation. Nevertheless, further analysis with Xnr3MO should be done to clarify endogenous role of Xnr3 in neural-epidermal boundary determination.

As described in Fig. 3, the mature-region of Xnr3 affected the neural patterning and the pro-region increased the activity of the mature region. Both cleavage of the pro-region and dimerization of the mature region are required for signaling activity of TGF-beta family members (Lopez *et al.*, 1992; Hawley *et al.*, 1995; Osada and Wright 1999). However, Xnr3 did not form homodimers, and the cleavage-mutant of Xnr3 sufficiently increased the expression of *Xbra* (Ezal *et al.*, 2000; Haramoto *et al.*, 2007). Furthermore, studies in zebrafish or cultured cells have shown that the pro-domain of nodal is not degraded soon after cleavage and indeed, that it plays a role in stabilizing the mature domain. The mature region alone has strong activity, but is also internalized and rapidly degraded in that state without the pro-region (Beck *et al.*, 2002; Le Good *et al.*, 2005). Based on these results, enhancement of the pro-region of Xnr3 may be due to stabilization of the mature region.

From the results of our study, we propose a novel mechanism of neural-epidermal boundary formation via specific cell migration

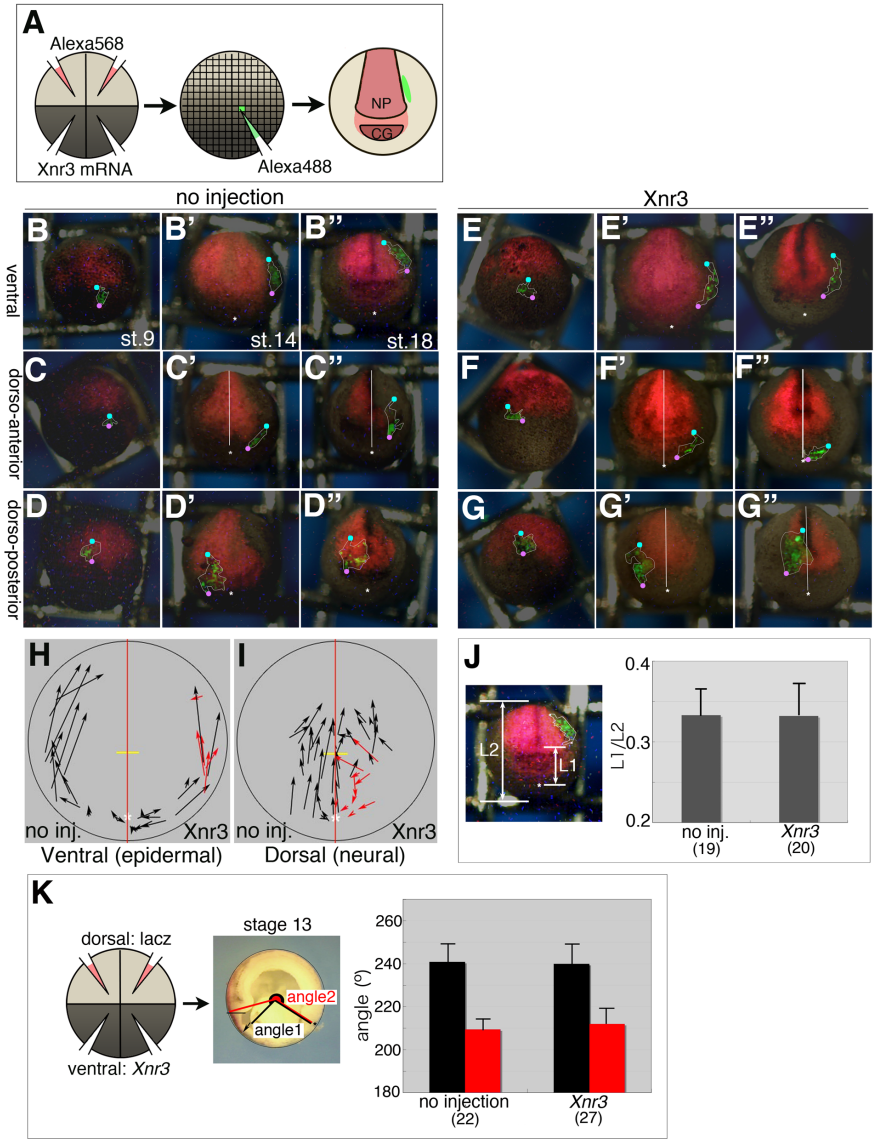


Fig. 5. Xnr3 specifically caused defects in neural-epidermal boundary cell migration. (A) *Schematic overview of the experiment. Alexa568-dextran (Red) and 100 pg of* Xnr3 *mRNA were injected into the DAP and VAP, respectively, of 4-cell embryos. After injecting Alexa488 (Green) into one cell in the epidermal-neural boundary region at the 128-cell stage, the injected embryos were examined at three points of stage: NP, neural plate; CG, cement gland.* **(B-G)** *Observation of migratory cells labeled with Alexa488 dextran during neurulation. Double-labeled embryos were injected with 0 pg (B-D) or 100 pg of* Xnr3 *mRNA, and then observed at stage 9 (B-G), stage 14 (B'-G'), or stage* 18 (B"-G"). *These embryos were all anteriorly viewed. B-B" and E-E" are embryos in that Alexa488 was ventrally injected. Similarly, C-C" and F-F," or D-D" and G-G" are injected into dorso-anterior (around animal pole) or into dorso-posterior (near marginal zone), respectively. The white line indicates the midline, and the asterisk marks the anterior end of the Alexa568-positive region. The Alexa488-positive domain is indicated by a dotted white line. The pink dots and the blue dots indicate the anterior end and the posterior end, respectively, of the Alexa488-positive area.* **(H,I)** *Summary of the results in regard to the migration of ventral cells (H) and dorsal (I) cells. In these diagrams, the result of normal embryo (left half) and* Xnr3 *injected embryo (right half) is shown. The red line and the short yellow line mark the midline of the embryo and anterior end of neural groove, respectively. The arrow indicates the movement of the migratory cells from Stage 14 to Stage 18. Black and Red arrows indicate mild changes and severe changes, respectively, in comparison with normal embryos.* **(J)** *Assessment of gastrulation defect by* Xnr3 *injection. In the left picture, L1 indicates the distance between the anterior end of the neural groove and the anterior end of the Alexa568 region, and L2 indicates the diameter of the embryo. L1/ L2 is shown in a bar graph. The error bars represent the standard error (SE). The numbers below the titles are the numbers of embryos examined.* **(K)** *The angle between the yolk plug and the anterior end of the neural plate (angle 1, black bars), and the angle between the yolk plug and the anterior end of the dorsal mesoderm (angle 2, red bars) are observed with half sections of the embryos. The results are shown in a bar graph. The error bars represent the standard error (SE). The numbers below the titles are the numbers of embryos examined.*

and cell shape change. Further research is now needed to clarify the molecular mechanism responsible for these events.

Materials and Methods

Embryos and microinjection

Xenopus laevis embryos were obtained by artificial fertilization. Fertilized eggs were dejellied with 4.6% L-cysteine hydrochloride at pH 7.8. Synthesized mRNAs were microinjected at a dose of 10 nl per embryo with a picoinjector PLI-100 (HARVARD APPARATUS). Embryos were cultured in 4.6% Ficoll until the gastrula stage and then in 10% Steinberg's solution.

Constructs and mRNAs

Capped mRNAs were synthesized using a SP6 mMESSAGE mMACHINE *in vitro* transcription kit (Ambion). pCS2-Xnr3, pCS2-pXtnr3, pCS2-mXtnr3 (Haramoto *et al*., 2004) and pCS2-β-galactosidase were used as templates for *in vitro* transcription.

Whole-mount in situ hybridization

The stages of development referred to in this study are according to the table of normal *Xenopus* developmental stages (Nieuwkoop and Faber, 1956). Antisense probes were synthesized with the following plasmids: pGEMT-krox20, p-engrailed-2(En2), p-xBF1, p-xRx1, p-xCG, p-sox2, p-slug, pSK5-six1; they were then labeled with digoxygenin or fluorescein. Embryos were fixed with MEMFA (0.1M MOPS (pH7.4), 2 mM EDTA, 1 mM MgSO4, 3.7% formaldehyde) and bleached with methanol/hydrogen peroxide solution. For lineage tracing, embryos were co-injected with 500 pg β-galactosidase mRNA and stained with Red-gal (Research Organics).

RT-PCR

Total RNAs were prepared from the excised anterior region of five embryos, and PCR was performed as described previously (Michiue *et al*., 2004). One non-injected sibling embryo served as a positive control. Ornithine decarboxylase (ODC) was used as a quantitative control.

Lineage tracing

To trace cell migration in the vicinity of the neural plate, 5 ng of Alexa568 was injected into the dorsal side of the animal pole at the 4-cell stage, and 5 ng of Alexa488 was injected into one cell located ventral to the animal pole at the 128-cell stage.

Phalloidin staining of actin

To mark the dorsal ectoderm, 5 ng of Alexa568 was injected into the dorsal side of the animal pole at the 4-cell stage. Late neurula-stage embryos were fixed with 4% formaldehyde, and then were transferred into 1% Triton solution in PBS. Subsequently dissected tissue was incubated with Alexa488-conjugated phalloidin to label F-actin, and cell shape was examined with a confocal microscope (Axiovert 100 or LSM-510).

Acknowledgements

We thank Dr. Moritoshi Sato for use of the confocal laser scanning microscope. This study was supported in part by Management Expenses Grants from the University of Tokyo and Grant-in-Aid for Scientific Research (C) in Japan Society for the Promotion of Science.

References

AGIUS E, OELGESCHLAGER M, WESSELY O, KEMP C, DE ROBERTIS E M (2000). Endodermal Nodal-related signals and mesoderm induction in *Xenopus*. *Development* 127:1173–1183.

BECK S, LE GOOD J A, GUZMAN M, BEN HAIM N, ROY K, BEERMANN F, CONSTAM D B (2002). Extraembryonic proteases regulate Nodal signalling during gastrulation. *Nat Cell Biol* 12: 981-985.

BOUWMEESTER T, KIM S, SASAI Y, LU B, DE ROBERTIS E M (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* 382: 595-601.

DURSTON A J, TIMMERMAN, J P, HAGE WJ, HENDRIKS H F, DE VRIES N J, HEIDEVELD M, NIEUWKOOP P D (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* 340: 140 – 144.

EZAL C H, MARION C D, SMITH W C (2000). Primary structure requirements for *Xenopus* nodal-related 3 and a comparison with regions required by *Xenopus* nodal-related 2. *J. Biol. Chem* 275: 14124 – 14131.

GLINKA A, WU W, ONICHTCHOUK D, BLUMENSTOCK C, NIEHRS C (1997). Head induction by simultaneous repression of Bmp and Wnt signalling in *Xenopus*. *Nature* 389: 517-519.

GLINKA A, WU W, DELIUS H, MONAGHAN A P, BLUMENSTOCK C, NIEHRS C (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* 391: 357-362.

HANSEN C S, MARION C D, STEELE K, GEORGE S, SMITH W C. (1997). Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3. *Development* 124: 483-492.

HARAMOTO Y, TANEGASHIMA K, ONUMA Y, TAKAHASHI S, SEKIZAKI H, ASASHIMA M (2004). *Xenopus* tropicalis nodal-related gene 3 regulates BMP signaling: an essential role for the pro-region. *Dev Biol* 265: 155-68.

HARAMOTO Y, TAKAHASHI S. ASASHIMA M (2007). Monomeric mature protein of Nodal-related 3 activates Xbra expression. *Dev Genes Evol* 217: 29-37.

HAWLEY S H, WUNNENBERG-STAPLETON K, HASHIMOTO C, LAURENT M N, WATABE T, BLUMBERG B W, CHO K W. (1995). Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. *Genes Dev* 9: 2923–2935.

HEMMATI-BRIVANLOU A, KELLY O G, MELTON D A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* 77: 283-295.

HERRMANN B G, LABEIT S, POUTSKA A, KING T R, LEHRACH H. 1990. Cloning of the T gene required in mesoderm formation in the mouse. *Nature* 343: 617-622.

HYDE C E, OLD R W (2000). Regulation of the early expression of the *Xenopus* nodal-related 1 gene, Xnr1. *Development* 127: 1221-1229.

JACOBSON A G, MOURY J D (1995). Tissue boundaries and cell behavior during neurulation. *Dev Biol* 171: 98-110.

JONES C M, KUEHN M R, HOGAN B L, SMITH J C, WRIGHT C V (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* 121: 3651-3662.

JOSEPH E M, MELTON D A (1997). Xnr4: a *Xenopus* nodal-related gene expressed in the Spemann organizer. *Dev Biol* 184: 367-372.

KURODA H, WESSELY O, DE ROBERTIS E M. 2004. Neural induction in *Xenopus*: requirement for ectodermal and endomesodermal signals via Chordin, Noggin, beta-Catenin, and Cerberus. *PLoS Biol* 2: E92.

KWAN K, KIRSCHNER M (2003). Xbra functions as a switch between cell migration and convergent extension in the *Xenopus* gastrula. *Development* 130: 1961-1972

LABONNE C, BRONNER-FRASER M (1998). Neural crest induction in *Xenopus*: evidence for a two-signal model. *Development* 125: 2403-2414.

LAMB T M, KNECHT A K, SMITH W C, STACHEL S E, ECONOMIDES A N, STAHL N, YANCOPOLOUS G D, HARLAND R M (1993). Neural induction by the secreted polypeptide noggin. *Science* 262: 713-718.

LE GOOD JA, JOUBIN K, GIRALDEZ A J, BEN-HAIM N, BECK S, CHEN Y, SCHIER A F, CONSTAM D B (2005). Nodal stability determines signaling range. *Curr Biol* 15: 31-36.

LEYNS L, BOUWMEESTER T, KIM S H, PICCOLO S, DE ROBERTIS E M (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* 88: 747-756.

LOPEZ A R, COOK J, DEININGER P L, DERYNCK R (1992). Dominant negative mutants of transforming growth factor-beta 1 inhibit the secretion of different transforming growth factor-beta isoforms. *Mol. Cell. Biol* 12: 1674–1679.

MASSAGUÉ J (1990). The transforming growth factor-beta family. *Annu Rev Cell Biol* 6: 597-641.

MARCHANT L, LINKER C, RUIZ P, GUERRERO N, MAYOR R (1998). The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. *Dev Biol* 198: 319-329.

MICHIUE T, FUKUI A, YUKITA A, SAKURAI K, DANNO H, KIKUCHI A, ASASHIMA M (2004). Idax, an inhibitor of the canonical Wnt signaling, is required for the anterior

neural structure formation in *Xenopus*. *Dev Dyn* 230: 79-90.

NIEUWKOOP P D, FABER J (1956). Normal table of *Xenopus laevis*. North-holland publishing company (Amsterdam, Netherland)

OSADA S I, WRIGHT C V (1999). *Xenopus* nodal-related signaling is essential for mesendodermal patterning during early embryogenesis. *Development* 126: 3229-3240.

PICCOLO, S., AGIUS, E., LEYNS, L., BHATTACHARYYA, S., GRUNZ, H., BOUWMEESTER, T. and DE ROBERTIS, E.M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* 397, 707-710.

RUIZ I ALTABA A (1992). Planar and vertical signals in the induction and patterning of the *Xenopus* nervous system. *Development* 116: 67-80.

SASAI Y, LU B, STEINBEISSER H, GEISSERT D, GONT L K, DE ROBERTIS E M (1994). *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* 79: 779-790.

SCHULTE-MERKER S, SMITH J C (1995). Mesoderm formation in response to Brachyury requires FGF signalling. *Curr. Biol* 5: 62–67

SMITH W C, HARLAND R M (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* 70: 829-840.

SMITH W C, KNECHT A K, WU M, HARLAND R M (1993). Secreted noggin protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm. *Nature* 361: 547-549.

SMITH W C, MCKENDRY R, RIBISI S J, HARLAND R M (1995). A nodal-related gene defines a physical and functional domain within the Spemann organizer. *Cell* 82: 37-46.

TADA M, SMITH J C (2000). Xwnt11 is a target of *Xenopus* Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* 127: 2227 -2238.

TAKAHASHI S, YOKOTA C, TAKANO K, TANEGASHIMA K, ONUMA Y, GOTO J, ASASHIMA M. (2000). Two novel nodal-related genes initiate early inductive events in *Xenopus* Nieuwkoop center. *Development* 127: 5319 – 5329.

VILLANUEVA S, GLAVIC A, RUIZ P, MAYOR R (2002). Posteriorization by FGF, Wnt, and retinoic acid is required for neural crest induction. *Dev Biol* 241: 289-301.

WILSON V, MANSON L, SKARNES WC, BEDDINGTON R S (1995). The T gene is necessary for normal mesodermal morphogenetic cell movements during gastrulation. *Development* 121: 877–886.

YOKOTA C, KOFRON M, ZUCK M, HOUSTON D W, ISAACS H, ASASHIMA M, WYLIE C C, HEASMAN J (2003). A novel role for a nodal-related protein; Xnr3 regulates convergent extension movements via the FGF receptor. *Development* 130: 2199-2212.

ZIMMERMAN L B, DE JESÚS-ESCOBAR J M, HARLAND R M (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86: 599-606.

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