**Xenopus tropicalis nodal-related gene 3 regulates BMP signaling: an essential role for the pro-region**

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**Abstract**

In vertebrates, nodal-related genes are crucial for specifying mesendodermal cell fates. Six nodal-related genes have been identified in *Xenopus*, but only one, nodal, has been identified in the mouse. The *Xenopus* nodal-related gene 3 (Xnr3), however, lacks the mesoderm-inducing activity of the other five nodal-related genes in *Xenopus*, and can directly induce neural tissue in animal caps by antagonizing BMP signals. In this study, we isolated three clones of the *Xenopus* (*Silurana*) *tropicalis* nodal-related gene 3 (Xtnr3) and analyzed their function. The Xtnr3 genes show high homology to Xnr3 and have the same activity. Southern blot and genomic PCR analyses indicate that the *X. tropicalis* genome has duplications in the Xtnr3 gene sequences and our three clones represent separate gene loci. We also found a partial clone of Xtnr3 that coded for the N-terminal part of its pro-region. Surprisingly, this sequence also induced neural tissue by antagonizing BMP signals, and its coded protein physically associated with BMP4 mature protein. Furthermore, we showed that the pro-region of Xnr5 has the same activity. Together, these findings indicate that the pro-region of nodal-related genes acts antagonistically towards BMP signals, which identifies a novel mechanism for the inhibition of BMP signaling.

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**Introduction**

Recent genetic studies have strongly implicated nodal-related genes in mesendoderm formation in vertebrates, and to date nodal-related factors remain the most likely candidates for mediators of endogenous mesendoderm induction. In *Xenopus*, six nodal-related genes have been identified: Xnr1, Xnr2, Xnr3, Xnr4, Xnr5, and Xnr6 (Jones et al., 1995; Joseph and Melton, 1997; Smith et al., 1995; Takahashi et al., 2000). Xnr3 differs from the other five in several respects. First, Xnr3 lacks a seventh cysteine, which is conserved among members of the TGF-β superfamily, and has a serine between the second and third cysteine, whereas all the others have a glycine in this position (Ezal et al., 2000). This feature suggests a structural difference between Xnr3 and the other Xnr genes. Second, while the other Xnr genes are regulated by VegT and β-catenin (Agius et al., 2000; Hyde and Old, 2000; Kofron et al., 1999; Takahashi et al., 2000; Xanthos et al., 2001), Xnr3 is activated by β-catenin signaling during gastrulation (McKendry et al., 1997). Third, in *Xenopus* animal cap assays, Xnr3 induces neural markers without forming mesendoderm (Hansen et al., 1997), whereas the other Xnr genes induce secondary axis formation (Smith et al., 1995). In addition, Xnr3 can block mesoderm induction by BMP and directly induce neural tissue in *Xenopus* animal caps. Based on these observations, Xnr3 has been categorized as an organizer BMP antagonist...
Materials and methods

Manipulations and microinjection of X. laevis embryos

X. laevis embryos were obtained by artificial insemination and cultured at 20°C in 100% Steinberg’s solution (SS) before gastrulation and in 10% SS after gastrulation. Fertilized eggs were dejellied using 1% sodium mercaptoacetate in 100% SS at pH 7.8. Microinjection was performed in 10% SS containing 8% Ficoll with synthesized mRNAs at a dose of 3 nl per embryo. Embryos were staged by comparison to the normal table of development of X. laevis (Nieuwkoop and Faber, 1956).

Construction of X. tropicalis cDNA library

Embryos from multiple crosses were used (one male and three females). Total RNA was isolated from stage 9 embryos using Trizol (Invitrogen). Polya RNA was obtained by oligo (dT) cellulose selection (Amersham Bioscience) and first-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). Library construction was performed using the ZAP II cDNA library system (Stratagene). A total of 1 × 10⁶ independent clones were obtained.

Cloning Xtnr3 cDNA

PCR products amplified from pdor-Xnr3 (Smith et al., 1995) using a previously described primer set (Takahashi et al., 2000) were labeled using Alkphos Direct (Amersham Bioscience) and used as a probe to screen the X. tropicalis blastula (stage 9) cDNA library. Three different X. tropicalis nodal-related gene 3 cDNA clones were identified and sequenced. The Xnr3 cDNA sequences were deposited in GenBank under accession numbers AB093327 for Xnr3-A, AB093328 for Xnr3-B and AB093329 for Xnr3-C.

Constructs and mRNAs

To generate pCS2+-Xnr3-A–C, and pXnr3ΔC, pBlue-scriptSK (-)-Xnr3-A–C and pXnr3ΔC were digested with EcoRI and XhoI to release Xnr3 cDNAs, which were cloned into the pCS2+ vector. These constructs contain 5’ and 3’ UTR. The construction of pCS2+-Xnr3 was performed by amplifying the ORF region of pdor-Xnr3 using the following primers: forward, 5’-GAGATGGCGATTTCTGAATCCAC-3’ and reverse, 5’-TGTTACATGTCCTTGAATCC-3’. PCR products amplified from pdor-Xnr3 were digested with EcoRI and XhoI, which were cloned into the pCS2+ vector. The construction of the truncated form of Xnr3, which encoded only the pro-region (pXnr3), was performed by amplifying the corresponding region of pdor-Xnr3 using the following primers: forward, 5’-GAGATGGCGATTTCTGAACCT-3’; and reverse, 5’-TTACCTTCTAAGTCTCCGAAATC-3’. For construction of pCS2+-pXnr3ΔC-6myc, pXnr3ΔC cDNA fragments were amplified by PCR from pCS2+-pXnr3ΔC with these primers: forward, 5’-CAAGCTTGTAT-

(Hansen et al., 1997; Smith et al., 1995). However, unlike Xnr3, overexpression of other BMP antagonists such as noggin, chordin, follistatin, Cerberus, and Gremlin (Bouwmeester et al., 1996; Hemmati-Brivanlou et al., 1994; Hsu et al., 1998; Lamb et al., 1993; Sasai et al., 1994, 1995; Smith and Harland, 1992; Smith et al., 1993; Zimmerman et al., 1996) induces secondary axis formation but not the formation of protrusions. Recently, it was also revealed that Xnr3 signaling via the FGF receptor regulates convergent extension movements but not neural induction (Yokota et al., 2003), suggesting that Xnr3 can regulate neural induction and convergent extension separately.

Although Xenopus laevis is commonly used as the model organism for vertebrate embryogenesis, it has a major drawback. At some stage in its evolution, the X. laevis genome has doubled, resulting in four copies of most genes instead of the usual two and making some genetic studies more difficult. Xenopus tropicalis is the only close relative of X. laevis with a diploid genome, which is the case for most other vertebrates. This organism could therefore be a useful tool for genetic analysis. Until now, however, few genes of X. tropicalis have been analyzed.

In this study, we isolated and analyzed the Xenopus (Silurana) tropicalis homolog of Xnr3 (Xtnr3). Unexpectedly, three clones with different sequences were identified as Xtnr3, suggesting that the advantage of the diploid genome may not apply to all genes. The Xnr3 genes were highly homologous to Xnr3 and have the same activity in both X. laevis and X. tropicalis embryos. We also found a partial clone of Xtnr3 that coded for the N-terminal part of its pro-region. Surprisingly, this sequence also induces neural tissue by antagonizing BMP signals, and its coded protein physically associates with BMP4 mature protein. Furthermore, we showed that the pro-region of Xnr5 has the same activity. Together, these findings indicate that the pro-region of nodal-related genes acts antagonistically towards BMP signals, which identifies a novel mechanism for the inhibition of BMP signaling.

Fig. 1. Sequence alignments of Xtnr3 and Xnr3. Identical sequences are shaded in medium gray. The open reading frame (ORF) is boxed. The ORF of Xnr3 and Xtnr3-A–C show high homology. However, there is some diversity among the 3’ untranslated regions (UTR).
The products were digested by ClaI and ligated into pCS2+ vector. To generate the truncated construct of Xnr5 that encodes only the pro-region (pXnr5), corresponding sequences of pNRRX-Xnr5 (Takahashi et al., 2000) were amplified with these primers: forward, 5'-GATATGGCTGTGCTAGGCTT-3'; and reverse, 5'-TTACCTCCTGTGTCGTCTTGC-3'. The products were ligated into the StuI site of pCS2+ vector.

For construction of the pro-region of cleavage mutant Xnr5 (pcmXnr5), the corresponding sequences of pNRRX-cmXnr5 (Onuma et al., 2002) were amplified with these primers: forward, 5'-CCCCTGCTGAGTATGCTGTGCTGACTCCTT-3' and reverse, 5'-GCTCTAGATTAGCCCCGTCGACTCCTT-3'. The products were digested with XhoI and XbaI, and ligated into the XhoI–XbaI site of the pCS2+ vector. For construction of pCS2+pXnr5-6myc, the pro-region of Xnr5 was amplified from pNRRX-Xnr5 with these primers: forward, 5'-GATATGGCTGTGCTAGGCTT-3'; and reverse, 5'-CTTGCCCTGTATTCTCCTG-3'. The PCR products were ligated into the blunt-ended ClaI site of pCS2+ vector. To construct pCS2+-BMP4-HA, HA epitope sequence (YPYDVPDYA) and an XhoI site (LE) were integrated between Q291 and Q292. All constructs were sequence-verified. mRNAs for microinjection were synthesized using SP6 mMESSAGE mMACHINE (Ambion) with templates from the following plasmids: pCS2+Xnr3-A; pCS2+Xnr3-B; pCS2+-Xnr3-C; pCS2+pXnr3AC; pCS2+Xnr3; pCS2+pXnr3; pCS2+pXnr3AC-6myc; pCS2+-BMP4-HA; pCS2+pXnr5; pCS2+pcmXnr5; pCS2+pXnr5-6myc; pdor-Xnr3 (Smith et al., 1995); pSP64T-BMP4 (Nishimatsu et al., 1992); and pCS2+β-gal (Takahashi et al., 2000).

Fig. 2. Temporal and spatial expression patterns of Xnr3s during X. tropicalis development. (A) Temporal expression was analyzed by RT-PCR. Lane numbers indicate stages according to Nieuwkoop and Faber (1956). The Xnr3 transcripts were first detected at the blastula stage (stage 8), and peaked through the mid-blastula (stage 8.5) to early gastrula stage (stage 10). ODC RT+ was used as an internal control. ODC RT− indicates no genomic DNA contamination. RT− indicates that reverse transcriptase was omitted from the RT reaction. (B–E) Whole-mount in situ hybridization shows the spatial expression patterns of the Xnr3s. (B) Vegetal view of stage 9 embryo; transcripts were detected around the dorsal region. (C) Vegetal and (E) dorsal views of stage 10 embryo; transcripts were restricted to the Spemann organizer region. (D) Vegetal view of stage 11 embryo; transcripts were decreased.
primers from 3 markers are indicated in kilobases. (B) Genomic PCR with gene-specific male).
extracted from the liver of six adult X. tropicalis individuals that were not siblings. Genomic DNAs were using pBluescriptSK (∼)
RNA was synthesized with T7 RNA polymerase (Roche)
exon-1 region of Xtnr3 primer pairs used were as follows: were performed according to Tanegashima et al. (2000). The RT-PCR and histology
The copy number of Xtnr3 was examined by Southern blot analysis. Genomic DNA from the liver of an adult male X. tropicalis was digested with EcoRI, HincII, or PstI. PCR fragments derived from the presumptive exon-1 region of Xtnr3 were used as probes. The positions of the DNA size markers are indicated in kilobases. (B) Genomic PCR with gene-specific primers from 3' UTR reveal all three forms, Xtnr3-A – C, present in multiple X. tropicalis individuals that were not siblings. Genomic DNAs were extracted from the liver of six adult X. tropicalis (five females and one male).

RT-PCR and histology

Total RNA isolation, RT-PCR, and histological analysis were performed according to Tanegashima et al. (2000). The primer pairs used were as follows: Xtnr3 (forward, 5'-AAGAAGCATCTCCTCAGCTGG-3'; reverse, 5'-TAGCTCAGCCAACTC-3'); X. tropicalis ODC (XtODC) (forward, 5'-GACATGTCAAGCCAGTTC-3'; reverse, 5'-TGGCCTCAGTTGCTTAT-3'); Xvent1 (forward, 5'-TTCCCTCAGCATGGTTCAAC-3'; reverse, 5'-GCATCTCCTTGGCATTTT-3'). The primer pairs for Xtnr3, ms-actin, EF-1 α, and NCAM were as described in the Xenopus Molecular Marker Resource (http://www.xenbase.org/WWW/Welcome.html). Ornithine decarboxylase (ODC) (Osborne et al., 1991) and Elongation factor-1 alpha (EF-1 α) were used as internal controls. Reverse transcriptase negative (RT-) reactions were included to indicate the absence of genomic DNA contamination.

Whole-mount in situ hybridization and lineage tracing

Whole-mount in situ hybridization analysis was performed according to Harland (1991). X. tropicalis embryos were obtained as described above and fixed in freshly prepared MEMFA [0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde] at room temperature for 2 h then stored at −30°C in methanol. DIG-labeled antisense RNA was synthesized with T7 RNA polymerase (Roche) using pBluescriptSK (−)-Xtnr3-A as a template. For lineage tracing, 150 pg of β-galactosidase mRNA was coinjected and visualized by Red-Gal (Research Organics) staining.

Genomic DNA extraction and Southern blot analysis

Genomic DNAs were prepared from liver of X. tropicalis and purified by phenol–chloroform extraction after proteinase K (Wako) treatment. The DNAs were digested with restriction enzymes overnight, and 15 μg was electrophoresed in 0.8% agarose gels, and blotted onto Hybond-N nylon membranes (Amersham Bioscience). The hybridization signals were detected using Alkphos Direct (Amersham Bioscience). PCR products for probes were amplified from pCS2+-Xtnr3-A with the following primer sets: Xtnr3 (forward, 5'-AAGAAGCATCTCCTCAGCTGG-3'; and reverse, 5'-GCTTTGACGGTATCGAATTCT-3'). These were designed to the exon-1 region, which was deduced from Xtnr3 cDNA sequences isolated by us and genomic sequences released by the U.S. Department of Energy Joint Genome Institute (JGI) (http://www.jgi.doe.gov/).

Genomic PCR analysis

Twenty nanograms of genomic DNAs from X. tropicalis and 1 ng of Xtnr3-A–C cDNAs were employed as templates for PCR amplification. PCR amplification were performed using r-Taq DNA polymerase (TaKaRa) with the following gene-specific primer sets, which were designed in 3' UTR: Xtnr3-A (forward, 5' -CTTTTAACCCACATTCACCAACTC-3'; and reverse, 5'-TGGGAGGAGAAGTGCTTCTG-3'); Xtnr3-B (forward, 5'-TCTCAGATTTCTTCATCCAT-3'; and reverse, 5'-CAGAGCCAGGTCAACGGAT-3'); Xtnr3-C (forward, 5'-AACACATCACAGACTTTCT-3'; and reverse, 5'-TCGCCATTTGCGCAACAT-3'). Genomic DNAs were extracted from six X. tropicalis individuals that were not siblings, and used as templates for PCR amplification. One of them (number 1, Fig. 3B) belonged to Stock B (Uehara et al., 2002), which was kindly provided by Dr. Yasuda (Nara Institute of Science and Technology, Nara, Ikoma-city, Japan), whose stocks were originally provided by Dr. Grainger (University of Virginia, Charlottesville, VA, USA). The other five (numbers 2–6, Fig. 3B) were wild-caught by a specialized animal dealer and have been maintained in our laboratory (Stock A; Uehara et al., 2002). PCR products were subcloned into the pGEM-T Easy Vector System (Promega) and characterized by sequencing.

Coimmunoprecipitation analysis

Injected embryos were harvested at stage 10 and lysed in lysis buffer [Dulbecco phosphate buffered saline (PBS) with 1% Triton X-100, 1 mM Pefablock SC (Roche) and 1 μg/ml leupeptin (Roche)]. After centrifugation, super-
natants were treated with protein G-Sepharose (Amersham Bioscience) bound with anti-c-Myc or anti-HA monoclonal antibody. The following antibodies were used for immunoprecipitation and Western blot analysis: IP, anti-c-Myc monoclonal antibody (9E10; Santa Cruz Biotechnology); detect, anti-c-Myc polyclonal antibody (A-14-G; Santa Cruz Biotechnology); IP, anti-HA polyclonal antibody HA-probe (Y-11; Santa Cruz Biotechnology); detect, anti-HA-Peroxidase High Affinity (Roche).

### Results

**Isolation of X. tropicalis nodal-related gene 3 (Xtnr3) cDNA**

To isolate Xtnr3, we screened an X. tropicalis cDNA library using X. laevis nodal-related gene 3 (Xnr3) cDNA as a probe. We isolated three clones, designated Xtnr3-A–C (Fig. 1), which all showed high homology with Xnr3 and whose open reading frame (ORF) nucleotide sequences are almost identical to each other. However, significant sequence diversity was found among the three clones in the 3′ untranslated region (UTR). Xtnr3 is unique among the Xenopus nodal-related genes in having several primary structure features that are different from the TGF-β superfamily consensus. First, Xnr3 lacks the last of the seven conserved cysteines in the mature protein. Second, all other superfamily members have a glycine residue located between the second and third cysteines, while Xnr3 has a serine residue at this site (Smith et al., 1995). Interestingly, these features were conserved in Xtnr3-A–C.

We also isolated a partial clone of Xtnr3. This clone was almost identical in sequence to Xtnr3-B, but without the coding sequence for the C-terminal part of the pro-region and the whole of the mature region, so we denoted this clone as pXtnr3ΔC. The pXtnr3ΔC protein therefore consisted of only the N-terminal 173 residues of the pro-region of Xtnr3. Since the transcripts of this clone were not detected by RT-PCR analysis, pXtnr3ΔC was thought to be an artificial clone, which we could use as a deletion construct for further investigation.

**Temporal and spatial expression of the Xtnr3 clones**

To determine the temporal expression of the Xtnr3 clones during normal development, RT-PCR analysis was carried out at various developmental stages. The Xtnr3s transcripts were first detected at the blastula stage (stage 8), and peaked through the mid-blastula (stage 8.5) to early gastrula stage (stage 10) (Fig. 2A). Next, we examined the spatial expression pattern of the clones using whole-mount in situ hybridization. At the late blastula stage, the transcripts were expressed in the dorsal region (Fig. 2B), and at the early gastrula stage, they were restricted to the Spemann organizer region (Figs. 2C,E). During gastrulation, transcript levels gradually decreased (Fig. 2D). These temporal and spatial expression patterns of the Xtnr3 clones resembled those of Xnr3.

**The X. tropicalis genome has duplications of the Xnr3 genes**

Embryos from multiple crosses (one male and three females) were used for construction of the cDNA library used here, introducing the possibility that this library could contain multiple alleles of every gene. To determine whether Xnr3-A–C represents polymorphisms in a single Xnr3 locus or separate gene loci, we first performed genomic Southern blot analysis. Genomic DNA derived from the liver of one male frog was digested with EcoRI, HincII, and PstI. A probe was designed from the presumptive exon-1 region, which was revealed from sequence comparison between Xnr3 cDNAs and genomic sequences from the JGI. Probe sequences contained no sites for the above-mentioned enzymes. Genomic Southern blot analysis of Xnr3 revealed multiple bands in every lane (Fig. 3A), indicating that the X. tropicalis genome has duplications of the Xnr3 genes. We then performed genomic PCR analysis with gene-specific primer sets to confirm the presence of all three forms, Xnr3-A–C, in multiple X. tropicalis individuals (Fig. 3B).
Fig. 4. Effects of injecting Xtnr3 mRNAs into X. tropicalis and X. laevis embryos. X. tropicalis embryos were injected into the animal pole of one blastomere at the two-cell stage with 150 pg of synthetic mRNA and cultured until stage 35. (A) Control injection with β-galactosidase (β-gal) mRNA, (B) Xnr3 mRNA, (C) Xtnr3-A mRNA, and (D) pXtnr3ΔC mRNA. X. laevis embryos were injected with 1 ng of synthetic mRNA in the same manner as X. tropicalis embryos and cultured until stage 35. (E) β-gal mRNA, (F) Xnr3 mRNA, (G) Xtnr3-A mRNA, and (H) pXtnr3ΔC mRNA. Xnr3-A and Xnr3 induced the same phenotype with anterior defects and tail-like protrusions in X. tropicalis and X. laevis embryos, but pXtnr3ΔC, lacking the mature region and the C-terminal part of the pro-region, did not. X. laevis embryos injected with pXtnr3ΔC (short form) developed a bump on the head. The blue and red arrowheads indicate the protrusion and the bump on the head, respectively. The black arrows indicate anterior defects. (I, J) Section of X. laevis embryo injected with Xnr3 mRNA into the animal pole. The protrusion contains mesenchymal tissue (arrow). The boxed area in I corresponds to J.
Genomic DNAs were extracted from six *X. tropicalis* individuals that were not siblings, and used as templates for PCR amplification. One of them (number 1, Fig. 3B) was a breeding *X. tropicalis*, originally descended from the colony of Dr. Grainger (University of Virginia, Charlottesville, VA, USA). The other five
The specificity of primer sets was examined by PCR amplification using Xtnr3-A–C plasmids as templates. All three forms of Xtnr3 were amplified from each genomic DNA with these gene-specific primer sets (Fig. 3B). We verified that the amplified fragments corresponded to each cDNA nucleotide sequence by sequencing subcloned PCR product (data not shown). These results indicate that Xtnr3-A–C represent separate unique genes rather than polymorphisms.

Fig. 6. pXnr3 (pro-region of Xnr3) mRNA induces neural but not mesodermal markers in animal caps, by blocking BMP signaling. (A) Control injection with β-gal mRNA, and (B) secondary axis induced by pXnr3 mRNA. X. laevis embryos were microinjected into the marginal zone of both ventral-vegetal blastomeres at the eight-cell stage with 500 pg of mRNA and cultured until stage 35. The red arrowhead indicates the secondary axis. (C) Four-cell-stage embryos were injected with 1 ng of mRNA into the animal pole of each blastomere. At the late blastula stage, injected and uninjected animal caps were dissected and cultured until sibling embryos reached stage 30. RT-PCR analysis shows that all Xnr3s and the pro-region of Xnr3 (pXnr3) induce the general neural marker NCAM and the cement gland marker XAG-1. The lack of ms-actin showed that neural induction occurred without a mesodermal intermediary. (D) Four-cell-stage embryos were injected with mRNA encoding BMP4 (1 ng) and BMP4 (1 ng) mixed with Xnr3, pXnr3, Xnr3-3-A, Xnr3-B, Xnr3-C, and pXtnr3-3AC (1 ng of each) into the animal pole of each blastomere. Animal cap explants were dissected at stage 9 and harvested at stage 10.5 for RT-PCR analysis. In each assay, expression of Xvent1 and Xmsx1, which are induced by BMP signaling, was blocked, suggesting that Xnr3, pXnr3, Xnr3s, and pXtnr3AC induce neural tissue by antagonizing BMP signaling. (numbers 2–6, Fig. 3B) were wild-caught individuals.
The pro-regions of Xnr3 and Xtnr3 act as BMP antagonists

Injection of pXtnr3ΔC, which codes for the N-terminal part of the pro-region, into the animal pole of two-cell embryos caused a bump on the developing head without anterior defects or protrusions (Fig. 4H). In addition, injection of Xnr3-A–C into the animal pole sometimes induced secondary axis formation (data not shown). Previous studies showed that Xnr3 can inhibit BMP signaling, but has little effect on secondary axis induction (Smith et al., 1995). To verify these data, X. laevis embryos were injected into the ventral marginal zone (VMZ). In this study, we showed that mRNAs synthesized from pCS2+-Xnr3 and pCS2+-Xtnr3-A–C were all able to induce a secondary axis, unlike the β-gal control (Figs. 5A–C; Table 2). In contrast, mRNA from p<em>dor-Xnr3</em> was not able to induce a secondary axis (data not shown) as previously reported (Smith et al., 1995). This result may be due to the different translational efficiencies of the two plasmids, pCS2+ and pdor. It is expected that pCS2+-Xnr3 has higher translational efficiency than pdor-Xnr3 and may therefore induce a secondary axis.

We also found that pXtnr3ΔC can induce a secondary axis when it is injected in the VMZ of eight-cell-stage X. laevis embryos (Fig. 5D; Table 2). A bump and a secondary axis induced by pXtnr3ΔC seem to represent the injected location of the embryo, that is, the animal pole or VMZ. To clarify these effects, pXtnr3ΔC was co-injected with a β-gal lineage tracer. Injected embryos were stained with red-gal to mark the pXtnr3ΔC-injected region and confirm targeting of mRNA. When X. laevis embryos were injected into the animal pole of one blastomere at the two-cell stage with pXtnr3ΔC mRNA (1 ng) and β-gal mRNA (150 pg), red-gal stain was seen on the head with a bump (Fig. 5E). On the other hand, when injected into the marginal zone of both ventral-vegetal blastomeres at the eight-cell stage with pXtnr3ΔC mRNA (500 pg) and β-gal mRNA (150 pg), a secondary axis was stained (Fig. 5F). These results indicate that the site of injection affects the resultant phenotype.

Xnr3 and Xtnr3 both induced formation of a partial but not complete secondary axis. Histological analysis confirmed that this secondary axis had a neural tube and otic vesicle but no eye, forebrain structure, or notochord (Figs. 5G,H). This finding is similar to that obtained from injection of a dominant negative form of the BMP receptor (Suzuki et al., 1994). In conclusion, the Xnr3-A–C clones induce a secondary axis with anterior defects and protrusions, in a manner similar to Xnr3.

It is known that TGF-β-related proteins consist of a pro-region and a mature region. Although many members of the TGF-β superfamily are expressed during early <em>Xenopus</em> embryogenesis, it has not been reported that the pro-region of the TGF-β superfamily exerts a biological function. To test the biological activity of the Xnr3 pro-region, pCS2+-pXnr3 mRNA, which encodes only the pro-region of Xnr3, was injected into the VMZ of <em>X. laevis</em> embryos.
This experiment showed that pXnr3 could induce a secondary axis in the same way as Xnr3, whereas the b-gal control did not (pXnr3; 23%, n = 91, Figs. 6A,B), suggesting that the pro-region of Xnr3 contributes functionally. A previous report suggested that Xnr3 inhibits BMP signaling, thereby inducing a secondary axis (Hansen et al., 1997). To confirm BMP inhibition in our experiments, BMP-4 was coinjected with Xnr3, pXnr3, Xnr3-A–C, and pXtnr3ΔC at a 1:1 ratio (1 ng, respectively) into animal caps. The animal cap assays showed that the expressions of Xvent1 and Xmsx1, which are directly induced by BMP4, were completely suppressed in each case (Fig. 6C). Thus, the pro-regions of Xnr3 and Xnr3 (pXnr3 and pXtnr3ΔC) have the ability to antagonize BMP signaling.
Next, we tested whether Xtnr3-A–C, pXnr3, and pXtnr3ΔC could directly induce neural markers in the animal caps (Fig. 6D). As predicted from the BMP inhibition experiments, each mRNA induced the general neural marker NCAM and the cement grand marker XAG-1, but there was no accompanying expression of the dorsal mesodermal marker ms-actin. The lack of ms-actin showed that the neural marker was induced directly, and not as a secondary effect of mesodermal tissue induction. These results led us to propose that the pro-region is sufficient for BMP antagonism of Xnr3 and Xtnr3.

The N-terminal part of the Xtnr3 pro-region interacts with BMP protein

The covalent heterodimerization between Xnr3 and BMP does not account for the inhibition of BMP signaling by pXnr3 or pXtnr3ΔC, because a cysteine residue in the mature region is essential for the covalent dimerization of TGF-β superfamily proteins (Brunner et al., 1992). Our results raise the possibility that Xnr3 and Xtnr3 inhibit BMP signaling through an alternative mechanism. Therefore, we examined whether pXtnr3ΔC physically associates with the BMP protein. mRNAs encoding Myc-tagged Xtnr3 were coimmunoprecipitated with BMP4 (Figs. 7A,B). These results indicate that BMP signaling is inhibited via an association between BMP4 and the N-terminal part of the Xnr3 pro-region.

The pro-region of Xnr5 inhibits BMP signaling

Previous results suggest that nodal inhibits BMP signaling (Yeo and Whitman, 2001). Our results raise the possibility that other nodal-related genes also inhibit BMP signaling in a manner similar to Xnr3. To examine the activity of the pro-regions of nodal-related genes, we generated two constructs of Xnr5, pXnr5 codes only for the pro-region of Xnr5, including the putative cleavage site. pcmXnr5 also codes only for the pro-region of Xnr5, but its cleavage site was changed to “GVDGG” to exclude the possibility of the cleavage site affecting the pro-region activity. This change is equivalent to that of cleavage mutant Xnr5 (cmXnr5) (Fig. 8A). As expected, pXnr5 and pcmXnr5 induced the expression of the general neural marker NCAM in animal cap explants without the expression of the dorsal mesodermal marker ms-actin (Fig. 8B), and inhibited the expression of Xvent1 and Xmsxx1, which are induced by BMP4 in animal caps (Fig. 8C). Furthermore, coimmunoprecipitation analysis showed that the pro-region of Xnr5 associates with BMP4 mature protein (Fig. 8D). These results suggest that the Xnr pro-region has a novel function to antagonize BMP signals via a physical interaction.

Discussion

The features of X. tropicalis nodal-related gene 3

X. laevis is a pseudotetraploid species, having four copies of most genes instead of the usual two. X. tropicalis is the only close relative of X. laevis with a diploid genome, and has drawn attention recently as a potential genetic model for early development. In this study, we isolated three variant clones of Xnr3. The diversity in their 3’ UTRs (Fig. 1) and their multiple loci (Fig. 3A) suggest that these three clones are separate genes resulting from duplications. In fact, genomic PCR results using gene-specific primers (Fig. 3B) show that all three forms, Xnr3-A-C, are present in multiple X. tropicalis individuals. Furthermore, we could find multiple clones that were nearly identical to the Xnr3 ORF sequences in the X. tropicalis genomic sequences, which have been recently released by the JGI, but they were not exactly the same as our clones. These observations suggest that there may have been polymorphisms between the X. tropicalis individual used for genome sequencing and the ones used in this study. Although we could not identify which genomic clones correspond to Xnr3-A-C in the ORF sequences, because they are too close to be distinguished from each other, they more diverse and they easily distinguished in the 3’ UTR region (Xnr3-A vs. Xnr3-B: 66%; Xnr3B vs. Xnr3-C: 58%; and Xnr3-C vs. Xnr3-A: 63%). We found sequences in the JGI database nearly identical to the 3’ UTRs of Xnr3-A [U1S14832.1; Identity = 317/321 (98%), TKS120942.1: 145/149 (97%), and ZWL293057.1: 205/208 (98%)], Xnr3-B [TKS59278.1: 246/249 (99%), and ZWL214892.1b: 363/365 (99%)], and Xnr3-C [AASO134100.1: 273/276 (98%), ZWK267298.1: 273/276 (98%), and ZWL254732.2y: 272/276 (98%)]. These results indicate that Xnr3-A, -B, and -C each have their own polymorphisms and they all represent separate loci rather than polymorphisms.

Xnr3 has the same activity in both X. laevis and X. tropicalis embryos

The injection of Xnr3-A-C, which all code for a protein containing the mature region, into the animal pole of X. tropicalis and X. laevis embryos induces anterior defects and protrusions in both, as does Xnr3 overexpression. Unlike other Xnr genes, Xnr3 has little ability to induce a secondary axis when it is ectopically expressed (Smith et al., 1995). In this study, we show that injection of mRNA synthesized from pCS2+Xnr3 and pCS2+Xnr3s, which may have high translational efficiency, induces secondary axis formation in VMZ. Injection of pXtnr3ΔC mRNA in the animal pole of X. tropicalis two-cell-stage embryos rarely induced a bump on the head, although a bump was frequently seen when pXtnr3ΔC mRNA was injected in the animal pole of X. laevis embryos. These differences could result from the difficulty in targeting mRNA due to small X.
tropicalis embryos, or a little difference of cell lineage between *X. laevis* and *X. tropicalis* in the animal region. Injection of *pXtnr3AC* mRNA in the VMZ of *X. tropicalis* eight-cell-stage embryos actually induces a secondary axis (data not shown), as it does when injected into *X. laevis* embryos. These results confirm that *Xnr3*, *Xnr3*, and their pro-region constructs function in *X. tropicalis* and *X. laevis* embryos, which supports the idea that the methods of manipulating embryos and evaluating gene function in *X. laevis* can be applied to *X. tropicalis*.

The *Xnr* pro-regions act as BMP antagonists

It has been reported that *Xnr3* inhibits BMP signals and induces the expression of neural markers in animal caps in the absence of the mesodermal marker *ms-actin* (Hansen et al., 1997). Recent studies have shown that some TGF-β-related proteins, which activate Activin/Nodal signaling, inhibit the function of BMP. A cleavage mutant of *nodal*, which acts in a dominant-negative fashion, inhibits Smad1 phosphorylation without Smad2 activation (Yeo and Whitman, 2001), and a cleavage mutant of *derrière* has neural-inducing activity without mesoderm induction (Eimon and Harland, 2002). We also observed that cleavage mutants of *Xnr2* and *Xnr5* suppress BMP signaling in animal cap assays (Y.O., S.T., K.T., C.Y., and M.A., unpublished observation), suggesting that the antagonism between BMP and *nodal* (including *Xnr3*) may be conserved among vertebrates. The results of these cleavage mutant studies may reflect covalent heterodimerization of BMP and other TGF-β-related proteins like Nodal and Derrière (Eimon and Harland, 2002; Yeo and Whitman, 2001). However, our results indicate that even the pro-region of *Xnr3* alone (*pXnr3*) demonstrates neural-inducing activity by antagonizing BMP signals. Furthermore, *pXtnr3AC*, which codes for about 65% of the N-terminal part of the pro-region, has the same activity. The studies using cleavage mutants do not exclude the possibility that the BMP-inhibiting activity of *Xnr3* requires the pro-region, since the mutations do not destroy the pro-region structure. Therefore, it is worth considering that the function of these cleavage mutants reflects not only the formation of covalent heterodimers but also an action of the pro-region. Although a previous report has shown that a truncated pro-region of *Xnr3* induces no NCAM expression in animal caps (Hansen et al., 1997), analysis of a series of mutants indicates the importance of the *Xnr3* pro-region. Activin–*Xnr3* fusion protein, which is made with the activin pro-region and cleavage site linked to the *Xnr3* mature region, is shown to have no activity in inducing the expression of NCAM (Ezal et al., 2000). These results also suggest that the pro-region of *Xnr3* is required for neural-inducing activity. To verify the relative neural-inducing potencies of the full-length and pro-region constructs of *Xnr3* and *Xnr3*, we performed animal cap assays with lower doses. When 500 pg of mRNA was injected in each embryo, the NCAM-inducing activities of *Xnr3* and *Xnr3-B* were still detectable, but those of *Xnr3-A*, *Xnr3-C*, *pXnr3*, and *pXtnr3AC* were not (data not shown). The difference between the activities of full-length constructs might result from their UTR sequences, which are more diverse than ORF sequences, or from their protein stability. It is difficult to correctly judge the potency of pro-region versus full-length constructs in neural induction. These results raise the possibility that although the pro-regions of *Xnr3* and *Xnr3* are capable of causing neural induction, the mature region may act cooperatively with them to exert the activity of *Xnr3* completely.

Since the TGF-β protein dimerizes covalently using a cysteine residue in the mature region (Brunner et al., 1992), our results do not account for covalent heterodimerization of *Xnr3* and BMP. An interaction between the pro-region and mature region of TGF-β superfamily members has been observed. The pro-region of TGF-β proteins is known to noncovalently associate with the mature region after precursor cleavage, so mature TGF-β cannot interact with its receptor and has no biological effects (Miyazono et al., 1988; Wakefield et al., 1988). In addition, the pro-region of Dorsalin, a BMP family member, forms stable complexes with its mature region and with mature Nodal (Constam and Robertson, 1999). Here, we provide direct evidence that the N-terminal part of the *Xnr3* pro-region is able to associate with BMP mature protein and inhibit its activity. Furthermore, we have shown that the *Xnr5* pro-region also antagonizes BMP signaling in the same manner as *Xnr3*. From these results, we propose that the pro-region of TGF-β superfamily members can interact with the other TGF-β-related mature proteins and modulate their activities. The pro-regions of TGF-β-related proteins have long been believed to modify the function of the mature region only, for example in promoting its dimerization (Gray and Mason, 1990), masking its activity (Gentry and Nash, 1990), or regulating its diffusibility (Cui et al., 2001). Our results now introduce the possibility that the pro-region of TGF-β-related proteins play more important and extensive roles in developmental processes than previously suggested.

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References


