Xmsx-1 homeobox protein as a downstream component of BMP signal in the early development of *Xenopus laevis*

Takamasa S. Yamamoto

CONTENTS

ABBREVIATIONS

ActRIA : activin receptor type IA

ActRIB : activin receptor type IB

ActRII : activin receptor type II

ADMP : anti-dorsalizing morphogenetic protein

ALK : activin receptor-like kinase

A/P : anterior/posterior

ARE : activin responsive element

Av.DAI : average dorsoanterior index

BMP : bone morphogenetic protein

BMPRIA : BMP receptor type IA

BMPRIB : BMP receptor type IB

BMPRII : BMP receptor type II

BSA : bovine serum albumin

cDNA : complementary DNA

DMZ : dorsal marginal zone

DNA : deoxyribonucleic acid

dnBMPR-IA : dominant negative BMP receptor IA

D/V : dorsal/ventral

EDTA : ethylenediamine-N,N,N',N'-tetraacetic acid

EGTA : O,O'-Bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid

eve : even-skipped

FGF : fibroblast growth factor

GST : glutathione S transferase

HA : hemagglutinin

HRP : horseradish peroxidase

MOPS : 3-morpholinopropanesulfonic acid

mRNA : messenger RNA

PBS : phosphate buffered saline

PCR : polymerase chain reaction

RNA : ribonucleic acid

RT-PCR : reverse transcription PCR

tBR : truncated BMP receptor IA

TGF-β : transforming growth factor

Tris : Tris(hydroxymethyl)aminomethane

UV : ultraviolet

VMZ : ventral marginal zone

GENERAL INTRODUCTION

A major question in vertebrate development is how the one cell egg gives rise to all cell types of the animal in the appropriate site on appropriate developmental timing. The first step of the complex biological process is a determination of three dimensional body axes, dorsal/ventral (D/V), anterior/posterior (A/P) and left/right (L/R). Next, pattern formation and cell fate specification along an individual axis occur and consequently, various organs form in the appropriate location. Thus, one of the central interests in developmental biology is elucidation of the mechanisms for the these events.

In African clawed frog, *Xenopus laevis*, the establishment of the primary body axes begins with fertilization of an egg (Moon and Kimelman, 1998). After fertilization, the cortex rotates an average of 30 degrees relative to the cytoplasm during the first cell cycle and the dorsal side of the embryo is determined at opposite side to the sperm entry point (Fig. 1). It is known that the series of events cause an accumulation of β-catenin protein in dorsal side of the embryo and subsequent D/V and A/P patternings begin (Larabell et al., 1997). These patterning events happen through blastula and gastrula stage and lead to the formation of Spemann's organizer, which is a signaling centre responsible for patterning the three germ layer, ectoderm, mesoderm and endoderm, in an upper dorsal blastopore lip of the gastrula embryo (Bouwmeester, 2001). The notion of "organizer" in amphibian embryo is based on the pioneering work by Hans Spemann and Hilde Mangold, who

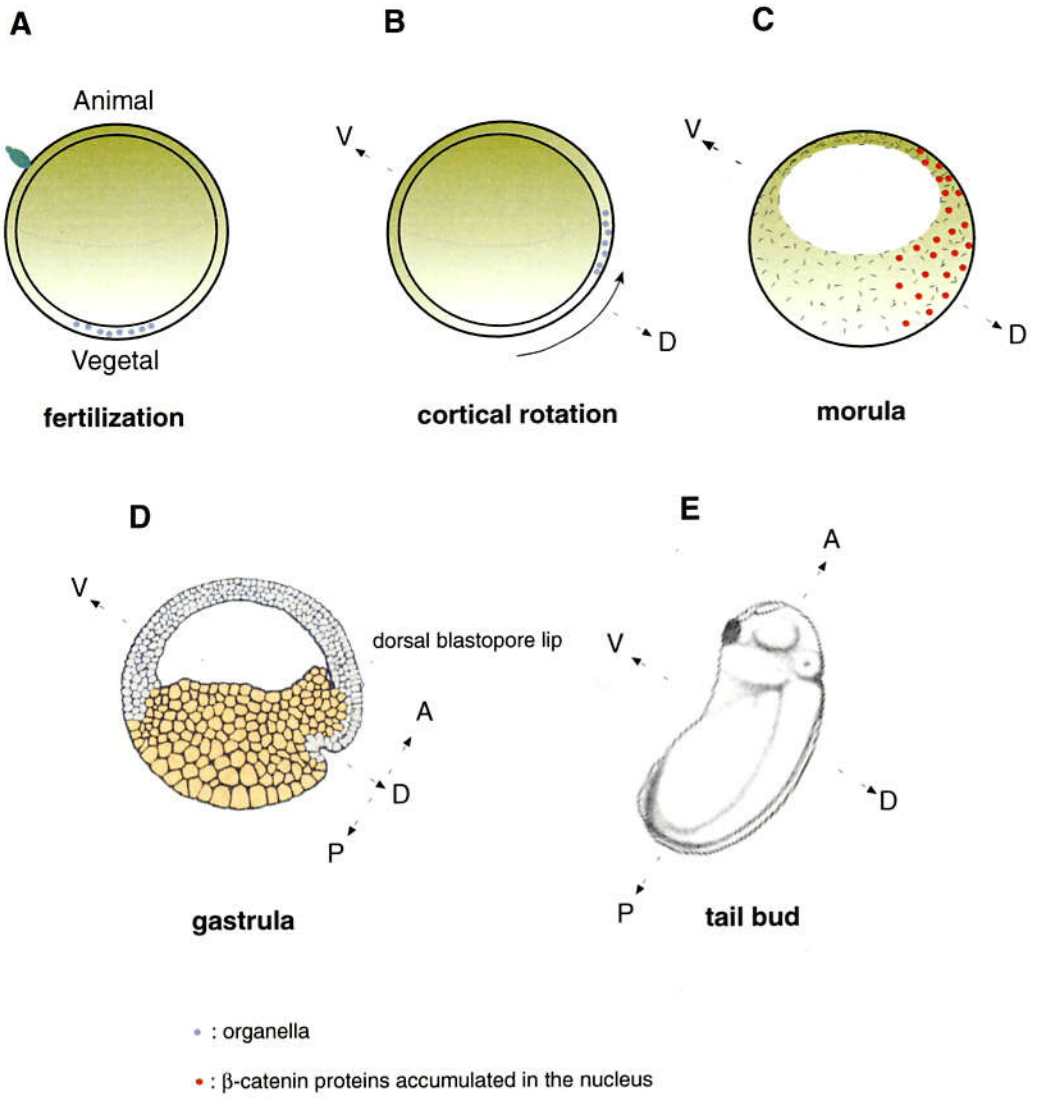


Fig. 1. Dorsal/ventral specification.

(A) In the unfertilized egg there is no evident D/V polarity. (B) After the fertilization the cortex rotates about 30 degrees relative to cytoplasm. The movement causes a 60- to 90-degree translocation of small organelles between the cortex and cytoplasm at the vegetalpole and D/V polarity is determined as above. (C) During the cleavage stage, β-catenin proteins accumulate in the nuclei of dorsal cells but not ventral cells. (D) By the gastrula stage Spemann's organizer is formed and gastrulation movement begins from posterior to anterior (from vegetal to animal pole). (E) Based on the pattern formation during these events all of the organs in tadpole are formed appropriately.

demonstrated that at the gastrula stage heterotopic transplantation of a dorsal blastopore lip from an albino newt into the ventral region of a pigmented newt resulted in the formation of a secondary body axis (Spemann and Mangold, 1924). Histological analysis revealed that the grafted albino cells predominantly differentiated into notochord but the other tissues including the somites and the nervous system in the secondary axis were derived from the pigmented host. From the non-cell-autonomous recruiting activity, the region was named "organizer". Spemann also proposed the existence of a distinct head and trunk organizer (Spemann, 1931). The transplantation of organizer of early gastrula induced complete secondary axis containing head, while the transplantation of organizer from late gastrulae induced only secondary trunk and/or tail. From these results, it was predicted that once organizer is formed by the patterning events in blastula stage, the almost embryonic body plan is carried out through the action of the organizer tissue and that the organizer field separates into head and trunk organizer region, which are qualitatively different, during gastrulation. Thus, to elucidate molecular nature of the organizer tissue and the molecular mechanisms separating head and trunk organizer are important to understand embryonic developmental process.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor β (TGF-β) superfamily which is one of the largest multifunctional families of signaling molecules, were discovered as proteins purified from a demineralized bovine bone extracts that induced ectopic cartilage and bone when implanted into rodents (Urist, 1965;

Wozney et al., 1988). Thereafter, these molecules were found to play a crucial role in a diverse array of developmental process including cellular survival, proliferation, morphogenesis, differentiation and apoptosis of multiple organisms (Hogan, 1996). In Xenopus, it was found that the genes for BMP subfamily members are expressed during embryogenesis and differentially regulated in developmental processes (Nishimatsu et al., 1992). Recent findings have revealed that two classes of members belonging to the TGF-β superfamily, nodal/activin related class and BMPs, regulate the embryonic pattern formation along D/V axis in vertebrate (Beddington and Robertson, 1999; Heasman, 1997). The first nodal/activin related class ligands has been shown to induce the formation of the dorsal mesoderm, including notochord and muscle, and neuralization of ectoderm. BMPs are shown to inhibit dorsalization of the mesoderm and neural induction from the ectoderm consequently inducing blood cells and epidermis, respectively (Hogan, 1996). BMPs are ubiquitously expressed in animal hemisphere and marginal zone of the blastula embryo (Hemmati-Brivanlou and Thomsen, 1995). From late blastula stage, BMP antagonists, chordin (Sasai et al., 1994), noggin (Smith and Harland, 1992) and follistatin (Hemmati-Brivanlou et al., 1994), begins to express in the organizer region and the BMP-4 expression fades in the dorsal side of mesoderm and ectoderm in gastrula (Hemmati-Brivanlou and Thomsen, 1995). Recent belief is that the gradient of BMP signaling activity along D/V axis acts as a morphogen (Kurata et al., 2000). My interest is what molecular mechanisms exert downstream of BMP to form the morphogen activity gradient. It is known that several homeobox genes, including

Xmsx-1 (Suzuki et al., 1997b), Xvent-1 (Gawantka et al., 1995) and Xvent-2 (Onichtchouk et al., 1996), are induced by activating BMP signaling in early Xenopus development. MSX-1, a mammalian counterpart of Xmsx-1, is well characterized in mouse organogenesis (Maas et al., 1996; Satokata et al., 2000). In this thesis I focused on the role and function of Xmsx-1 as a BMP target gene in early development of *Xenopus* embryo.

In Part I, requirement of Xmsx-1 in the ventralizing signal by BMP was investigated using a dominant-inhibitory form of Xmsx-1. Epistatic relationship between Xmsx-1 and another ventralizing homeobox protein Xvent-1 was also shown. In Part II, I proposed that BMP/Xmsx-1 signal is involved in the regulation of head formation as well as D/V axis pattern formation. I also investigated the mechanisms of the interaction between Xmsx-1 and nodal signaling in the head formation.

PART I

Requirement of Xmsx-1 in the BMP-triggered ventralization of *Xenopus* embryos

Summary

Signaling triggered by polypeptide growth factors leads to the activation of their target genes. Several homeobox genes are known to be induced in response to polypeptide growth factors in early *Xenopus* development. In particular, *Xmsx-1*, an amphibian homologue of vertebrate *Msx-1*, is well characterized as a target gene of BMP. Here, using a dominant-negative form of Xmsx-1 (VP-Xmsx-1), which is a fusion protein made with the virus-derived VP16 activation domain, I have examined whether Xmsx-1 activity is required in the endogenous ventralizing pathway. VP-Xmsx-1 induced a secondary body axis, complete with muscle and neural tissues, when overexpressed in ventral blastomeres, suggesting that Xmsx-1 activity is necessary for both mesoderm and ectoderm to be ventralized. I have also examined the epistatic relationship between Xmsx-1 and another ventralizing homeobox protein Xvent-1 and show that Xmsx-1 is likely to be acting upstream of Xvent-1. I propose that Xmsx-1 is required in the BMP-stimulated ventralization pathway that involves the downstream activation of *Xvent-1*.

Introduction

Cell-to-cell interactions during development are regulated by a number of polypeptide growth factors which are secreted from one type of cell and act on other types of target cells to determine their fate (Boonstra et al., 1995; Hogan, 1996; Nusse and Varmus, 1992; Sive, 1993; Szebenyi and Fallon, 1999). The activities of these factors are known to be mediated through membrane receptors, intracellular signaling factors, and nuclear transcriptional factors and their coactivators in the target cells (Derynck et al., 1998; Gotoh and Nishida, 1995; Jans and Hassan, 1998; Miller and Moon, 1996). As a result of signal activation, information from individual growth factors leads to the induction of their target genes, including homeobox transcription factors (Botas, 1993), which in turn direct secondary gene activation or repression. Recent studies have revealed that some of these transcriptional regulators are immediate early genes responding to polypeptide growth factors' stimuli and have critical roles in cell differentiation during early development (Ladher et al., 1996; Suzuki et al., 1997b).

In *Xenopus*, dorsal mesoderm inducing signals represented by activin, Vg1, and nodal (Jones et al., 1995; Kessler and Melton, 1995; Smith et al., 1990; Thomsen et al., 1990), have been shown to induce a number of transcriptional factors including a homeobox protein called goosecoid (Cho et al., 1991) and the LIM-homeo domain protein Xlim-1 (Taira et al., 1992), both of whose expression is restricted to the organizer region. Thus, these genes are called "organizer-specific" genes. Similarly, ventral inducers of

BMP have been shown to activate the transcription of several homeobox genes; *Xvent-1/PV.1* (Ault et al., 1996; Gawantka et al., 1995), *Xvent-2/Xom/Vox/Xbr-1* (Ladher et al., 1996; Onichtchouk et al., 1996; Papalopulu and Kintner, 1996; Schmidt et al., 1996), *Xmsx-1* (Maeda et al., 1997; Suzuki et al., 1997b), and *mouse Dlx5* (Miyama et al., 1999). It is interesting to note that all these genes are not only induced by BMP-4 but also have the capacity to ventralize embryos when ectopically expressed in the dorsal blastomeres, mimicking the effect of BMP-4 (Ault et al., 1996; Gawantka et al., 1995; Ladher et al., 1996; Maeda et al., 1997; Miyama et al., 1999; Onichtchouk et al., 1996; Schmidt et al., 1996; Suzuki et al., 1997b). Among these, Msx-1 (Boncinelli, 1997; Davidson, 1995), the mouse counterpart of Xmsx-1, has a well characterized role in organogenesis. In the mouse, Msx-1 and Msx-2 mediate epithelial-mesenchymal interactions in tooth induction; Msx-1 is induced in dental mesenchyme by epithelially expressed BMP-4 (Vainio et al., 1993). BMP-4 also requires Msx-1 to induce its own expression in mesenchyme, suggesting a genetic cascade in tooth development: BMP-4 (epithelium)--Msx-1(mesenchyme)--BMP-4 (mesenchyme) (Chen et al., 1996b). In addition, Msx-1 is induced by BMP-2 in the neural crest in rhombomeres 3 and 5 (Graham et al., 1994). The expression of BMP-2 in the hindbrain is implicated in the induction of apoptosis through the activity of Msx-1, which eliminates neural crest cells from the rhombomeres (Graham et al., 1994). In addition to tooth development and the establishment of rhombomere identity, the cascade also seems to be present in limb formation (Vogel et al., 1995) and craniofacial development (Foerst-Potts and Sadler, 1997).

Thus, a growing body of experimental evidence suggests that Msx genes are both targets and essential mediators of BMP signaling.

In *Xenopus* embryogenesis, Xmsx-1 is expressed in both ventral mesoderm and ectoderm, and its expression overlaps with that of BMP-4 (Maeda et al., 1997; Suzuki et al., 1997b). In fact, *Xmsx-1* is induced in animal cap cells as an immediate early gene, in response to BMP receptor activation (Suzuki et al., 1997b). In experiments where a dominant-negative BMP receptor was used to block the BMP signal, ectopically expressed Xmsx-1 acted downstream of the BMP receptors to restore the BMP signal (Suzuki et al., 1997b), suggesting that Xmsx-1 has a major role in BMP signaling.

In this study, I generated functional derivatives of Xmsx-1 by constructing a cDNA that encodes an Xmsx-1 protein fused with the transactivation domain of virus-derived VP16 (Friedman et al., 1988) or with the repressor domain of the *Drosophila even-skipped* protein (Han and Manley, 1993). My functional analysis using early *Xenopus* embryos showed that VP-Xmsx-1 but not eve-Xmsx-1 inhibits the effect of overexpressed Xmsx-1, suggesting that Xmsx-1 is likely to be a transcriptional suppressor. Using the dominant-negative Xmsx-1, I also found that Xmsx-1 is required for the ventralization triggered by BMP. Furthermore, I demonstrated that Xmsx-1 acts upstream of another ventralizing homeobox gene, *Xvent-1*. Finally, I propose that there is a hierarchy of homeobox genes in the ventralizing pathway responding to BMP.

Experimental procedures

Constructs

Activating (VP-Xmsx-1) and repressing (eve-Xmsx-1) forms of Xmsx-1 were created by replacing the N-terminal 136 aa of Xmsx-1 with the 81 aa activation domain of VP16 protein (VP16 AD) to make VP-Xmsx-1 or the 269 aa repressor domain of the *Drosophila* even-skipped protein (eve RD) to make eve-Xmsx-1. The pCS2VP16 and pCS2eve plasmids, which contain the VP16 activation domain and the even-skipped repressor domain, respectively, were gifts from Ken W. Y. Cho. The negative control (ΔNXmsx-1) construct was created by fusing an initiation codon, ATG, to the sequence encoding the C-terminal half of the Xmsx-1 protein (amino acids 137-275). VP-Xvent-1 cDNA (a gift from Christof Niehrs) was subcloned into the pCS2+ plasmid.

Manipulation of embryos

Unfertilized eggs were collected and fertilized in vitro as described previously (Suzuki et al., 1997a). Embryos were dejellied using 3% cysteine and washed with water several times. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). The animal cap, ventral marginal zone (VMZ), and dorsal marginal zone (DMZ) were dissected at stage 8 for the animal cap and stage 10 for the marginal zones, and cultured in 0.1% BSA/1x Steinberg's solution (Asashima et al., 1990).UV irradiation of embryos was performed with a Stratalinker

(Stratagene). 40 min after fertilization embryos were placed in water on quartz watch glass and irradiated.

Microinjection of synthetic mRNA

Each construct was linearized, and capped mRNAs were synthesized in vitro using the mMESSAGE mMACHINE sp6 kit (Ambion). Synthesized RNAs were injected into the animal pole or marginal zone of 2- or 4-cell stage embryos, respectively, in 3% Ficoll/0.1x Steinberg's solution as described previously (Suzuki et al., 1997a).

Reverse transcription-polymerase chain reaction (RT-PCR)

To detect molecular marker expression in animal caps and marginal zone explants, total RNAs were extracted using TRIzol reagent (GIBCO/BRL) according to the manufacturer's instructions. Extracted RNAs were subjected to RT-PCR. Primer sequences for each marker were as follows: *chordin*, a dorsal organizer marker, upstream, 5'-CAGTCAGATGGAGCAGGATC-3' and downstream, 5'-AGTCCCATTGCCCGAGTTGC-3'; *Xmyf-5*, dorsolateral mesodermal marker, upstream, 5'-CAGAATGGAGATGGTAGATAGC-3' and downstream, 5'-AGCCTGGTTCACTTTCTTTAGC-3'; *Xwnt-8*, a ventrolateral mesodermal marker, upstream, 5'-GTTCAAGCATTACCCCGGAT-3' and downstream, 5'-CTCCTCAATTCCATTCTGCG-3'; *Zic-3*, an early neural marker, upstream, 5'-TACTGCTCAGCTCATTCATG-3' and downstream, 5'-AATTGGCATATCCAGAAGCC-3'; *NCAM*, a late neural marker,

upstream, 5′-GCCTGTAGAATTACAATGCTG-3′ and downstream, 5′-AGCATCTTGGCTGCTGGCATT-3′; *Xmsx-1*, upstream, 5′-ACTGGTGTGAAGCCGTCCCT-3′ and downstream, 5′-TTCTCTCGGGACTCTCAGGC-3′; *Xvent-2*, upstream, 5′-GGACTATACTAAAGGCTGGA-3′ and downstream, 5′-ATTACTCATAGAATATACAC-3′; *BMP-4*, upstream, 5′-CATCATGATTCCTGGTAACCGA-3′ and downstream, 5′-CTCCATGCTGATATCGTGCAG-3′; *xGATA-2*, upstream, 5′-GCGGACTCTACTACAAATTG-3′ and downstream, 5′-AAAGGTGCCAAGTGACCCAT-3′. Primer sequences of *goosecoid*, an organizer specific marker, *histone H4*, an internal input control, and *Xvent-1* were as previously described (Iemura et al., 1998).

Histology

Embryos were fixed in 4 % paraformaldehyde/PBS at the tailbud stage. After being embedded in paraffin, the embryos were sectioned and stained with hematoxylin and eosin.

Results

VP-Xmsx-1 inhibits Xmsx-1 activity

Although it was previously reported that Xmsx-1 is sufficient to exert a BMP-like activity, namely the ventralization of embryos, when dorsally overexpressed (Maeda et al., 1997; Suzuki et al., 1997b), the requirement for Xmsx-1 in the ventralization pathway triggered by BMP was not clear. I, therefore, attempted to inhibit the function of endogenous Xmsx-1 by using functional variants of Xmsx-1. I generated three cDNAs that encode (a) the N-terminally deleted form of Xmsx-1, (b) a fusion protein of Xmsx-1 with the transactivation domain derived from VP16 (Friedman et al., 1988), and (c) the repressor domain of the *even-skipped* (*eve*) gene product (Han and Manley, 1993) (Fig. I-1). I expected to give useful information about the endogenous role of Xmsx-1 as a transcriptional activator or repressor by using these constructs to force the activation or inactivation of Xmsx-1 target genes, a strategy that has been productive in other instances (Conlon et al., 1996; Ferreiro et al., 1998; Onichtchouk et al., 1998). Therefore, mRNAs were synthesized from these cDNAs and injected into early *Xenopus* embryos to ascertain their functions in terms of ventralizing activity. As reported previously, Xmsx-1 mRNA alone induced a ventralized phenotype, with an average dorsoanterior index (Av.DAI) of 3.2 (n=27) (Kao and Elinson, 1988), when overexpressed in dorsal blastomeres, which resembles the phenotype induced by BMP overexpression (Fig. I-2A) (Maeda et al., 1997; Suzuki et al., 1997b). First, I examined whether any of these artificial variants of Xmsx-1 could suppress

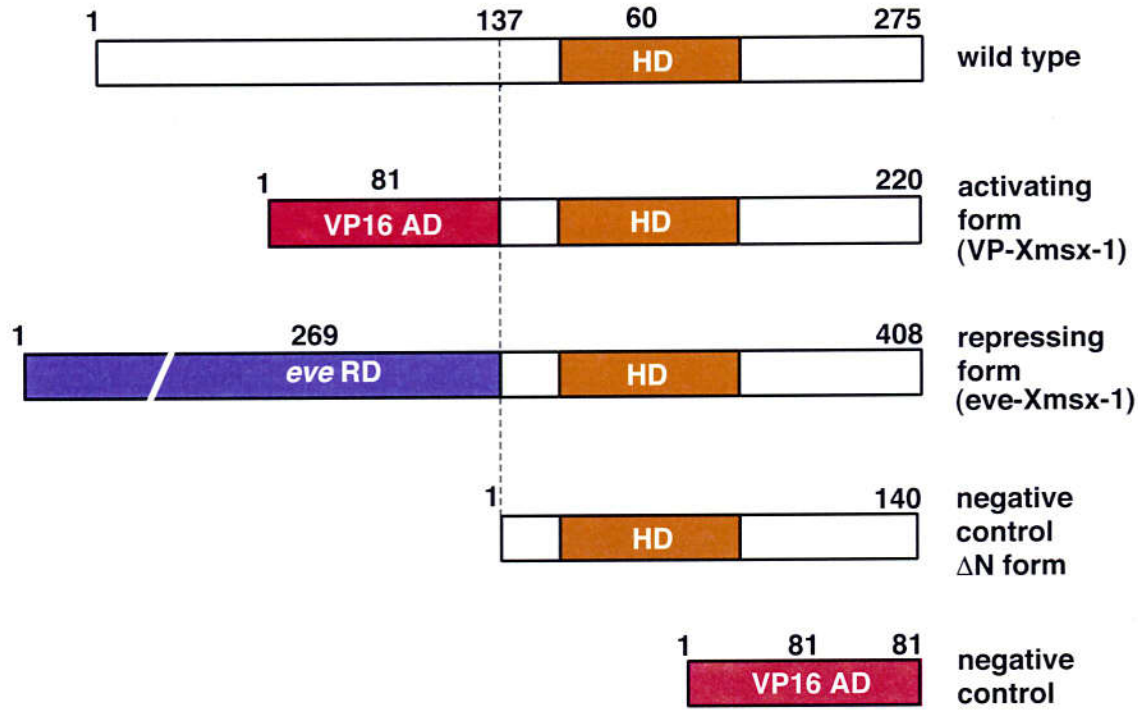


Fig. I-1. Schematic representation of wild-type and mutated Xmsx-1 molecules. The amino acid numbers of several sites are shown above each drawing of the predicted protein and the homeodomain is indicated by the orange box (HD). The top drawing represents wild-type Xmsx-1 protein. Activating (VP-Xmsx-1) and repressing (eve-Xmsx-1) forms of Xmsx-1 were constructed by replacing the N–terminal 136 amino acids of Xmsx-1 with the 81 amino acids of the VP16 activation domain (VP16 AD) or the 269 amino acids of the repression domain of the *even-skipped* protein (eve RD), respectively. The VP16 activation domain alone and ΔNXmsx-1 were used as negative control constructs.

the ventralized phenotype caused by the overexpression of wild-type Xmsx-1. The ventralizing effect of wild-type Xmsx-1 was almost completely inhibited by the coexpression of VP-Xmsx-1 (Av.DAI 4.7, n=29) (Fig. I-2B), while no or slightly enhanced ventralization was observed by coexpression of eve-Xmsx-1 (data not shown). To evaluate the specificity of VP-Xmsx-1, I used two other homeobox genes, *Msx-2* and *Xdll-3,* whose primary structures are similar to Xmsx-1 (amino acid sequence identity in homeodomain and overall structure, Msx-2: 100% and 54.9%, Xdll-3: 56.7% and 25.8%, respectively). Each mRNA was coinjected with VP-Xmsx-1 mRNA. Msx-2 is the most homologous to Msx-1 of all known gene products, and both amino acid sequences are completely identical in their homeodomains. Xdll-3 (Papalopulu and Kintner, 1993) is a *Xenopus* homeobox protein most similar to Dlx-5 in other vertebrates, which has recently been identified as a target gene of BMP-2/4 (Miyama et al., 1999). I also showed that overexpression of Xdll-3 (Fig. I-2C) or mouse Msx-2 (mMsx-2, data not shown) in dorsal blastomeres resulted in the ventralization of the embryo, mimicking the effect of BMP and Xmsx-1, and demonstrating that they are also ventralizing homeobox proteins. VP-Xmsx-1 did not rescue the Xdll-3-induced ventralized embryo to any extent (Fig. I-2D), although it did show a weak rescuing ability for the mMsx-2-induced ventralization (data not shown), indicating that VP-Xmsx-1 is a specific inhibitor of Xmsx-1, but leaving the possibility of weak cross-reaction with Msx-2 target sequences open. I also found that VP-Xmsx-1 does not rescue the ventralized phenotype caused by Xvent-1 or -2 (Gawantka et al., 1995; Onichtchouk et al., 1996) (See discussion on the

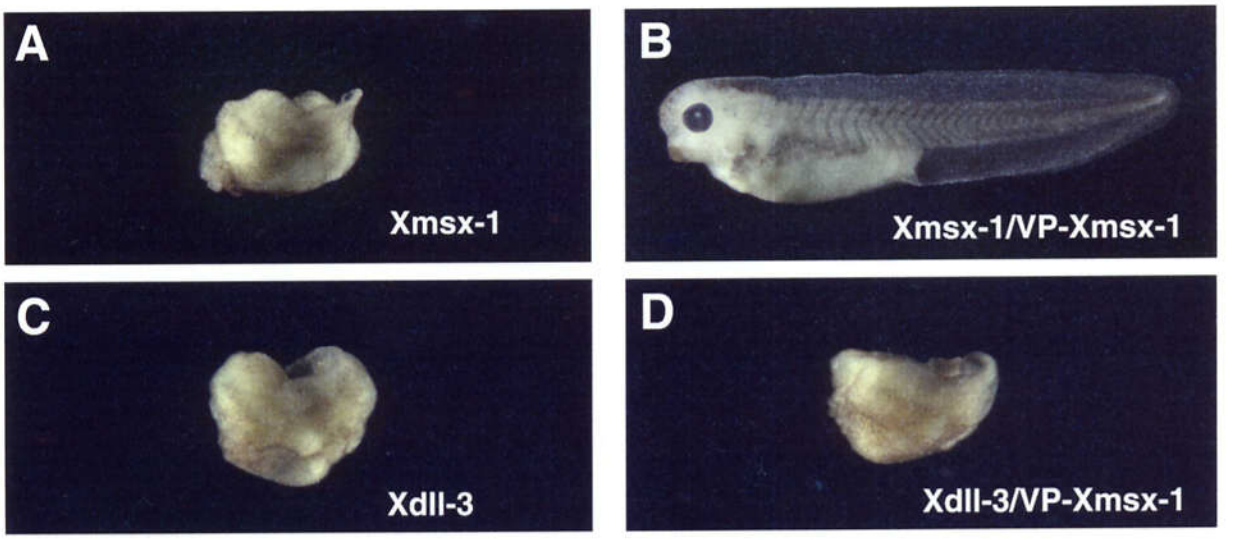


Fig. I-2. VP-Xmsx-1 specifically antagonized Xmsx-1.
(A, B) 500 pg of Xmsx-1 mRNA either alone (A) or with 100 pg of VP-Xmsx-1 mRNA (B) was injected into the equatorial region of two dorsal blastomeres of 4-cell stage Xenopus embryos. (C, D) 50 pg of Xdll-3 mRNA either alone (C) or with 500 pg of VP-Xmsx-1 mRNA (D) was injected in the same manner. VP-Xmsx-1 rescued the ventralization by Xmsx-1 but not by Xdll-3.

epistatic relationship between Xmsx-1 and Xvents below). Neither the VP16 activation domain nor the N-terminally deleted form of Xmsx-1, when expressed alone, had much effect on the ventralization induced by Xmsx-1 (data not shown). Thus, the inhibitory activity of VP-Xmsx-1 against wild type Xmsx-1 is specific to the fusion construct, indicating that the target genes recognized by the C-terminal region of Xmsx-1, including the homeobox, were subjected to forced activation. These data suggest that the VP-Xmsx-1 acts in a dominant-negative manner to inhibit native Xmsx-1.

Xmsx-1 is a transcriptional repressor

I next used VP-Xmsx-1 mRNA to inhibit the endogenous Xmsx-1 activity. *Xmsx-1* is expressed in the ventral half of embryos at the early gastrula stage (Maeda et al., 1997; Suzuki et al., 1997b). Thus, it is believed to be a downstream mediator of BMP-4, which is also expressed in ventral mesoderm and ectoderm (Hemmati-Brivanlou and Thomsen, 1995). Overexpression of VP-Xmsx-1 in the ventral blastomeres caused the development of a partial secondary body axis (85.7%, n=28)(Fig. I-3Ab). Hematoxylin and eosin staining showed that the secondary body axis contained muscle and neural tissues but no recognizable notochord (Fig. I-3C). This secondary body axis is similar to that induced by ventral overexpression of a dominant-negative BMP type I receptor (dnBMPR-IA) (Maeno et al., 1994; Suzuki et al., 1994). Thus, it appeared that as a consequence of the inhibition of endogenous Xmsx-1 by VP-Xmsx-1, the ventral mesoderm and ectoderm were respecified to become the respective

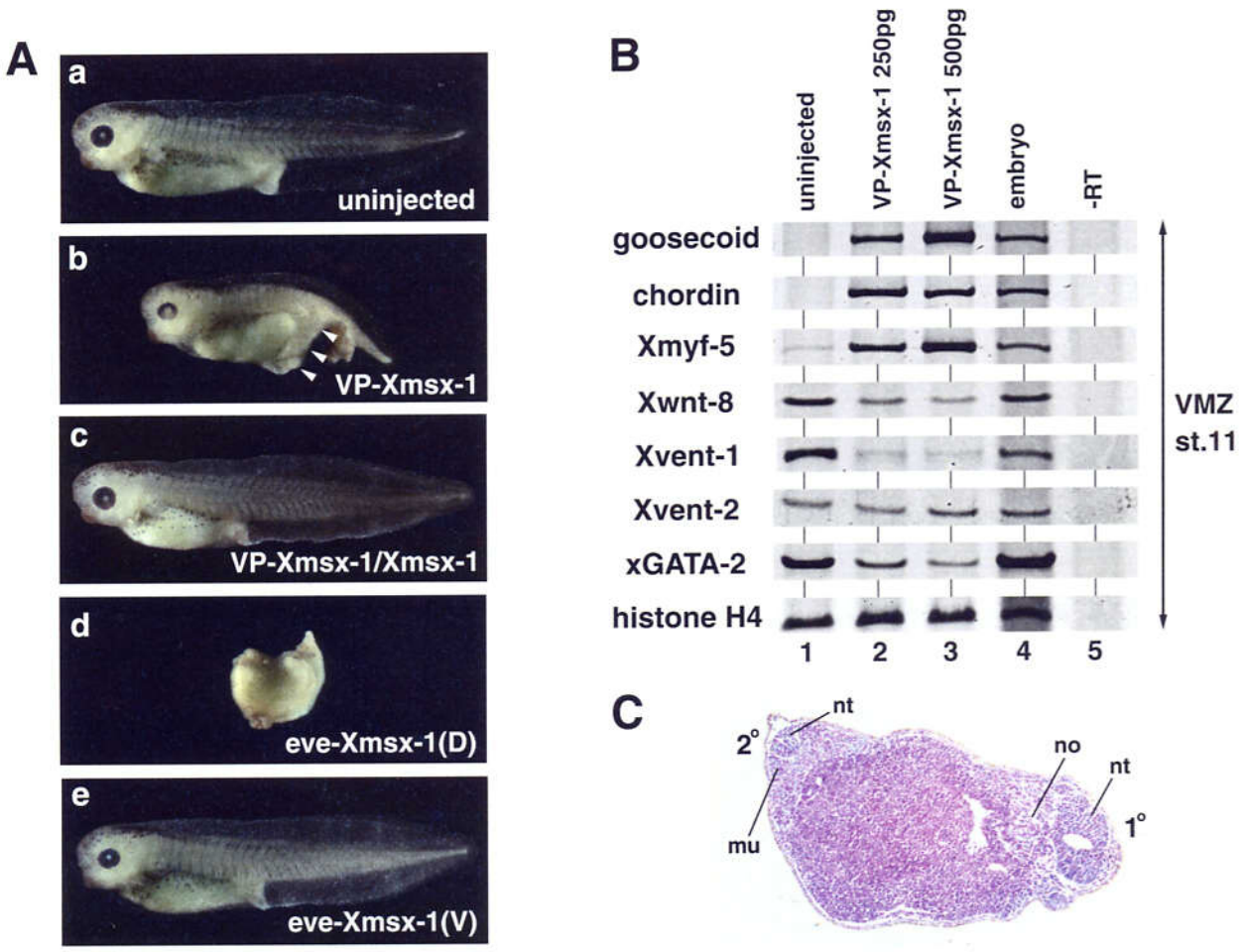


Fig. I-3. VP-Xmsx-1 dorsalized the embryo.
(A) Phenotypes of embryos microinjected at the 4-cell stage into the equatorial region of two ventral (b, c, e) or dorsal (d) blastomeres with 250 pg of VP-Xmsx-1 mRNA (b), a mixture of 250 pg VP-Xmsx-1 and 500 pg wild-type Xmsx-1 mRNA (c) or 500 pg of eve-Xmsx-1 mRNA (d, e). (a) uninjected embryo. A secondary body axis is indicated by the white arrowheads. (B) Analysis of gene expression in the ventral marginal explant by RT-PCR. Embryos were either uninjected (lanes 1, 4, 5) or injected with 250 pg (lane 2) or 500 pg (lane 3) of VP-Xmsx-1 mRNA into the equatorial region of the two ventral blastomeres at the 4-cell stage. Ventral marginal zones (VMZ; lanes 1, 2, 3) were excised and explanted at the early gastrula stage and incubated until sibling embryos reached stage 11. RNA extracted from each explant was analyzed by RT-PCR. Lane 4 shows the expression of each marker in whole embryos and lane 5 shows the control reactions with no RT step. (C) Cross section of the embryos ventrally injected with 250 pg of VP-Xmsx-1 mRNA. Primary (1) and secondary (2) axis structures were observed. nt: neural tube, no: notochord, mu: muscle

dorsal tissues. To confirm this possibility, I coinjected wild-type Xmsx-1 mRNA with VP-Xmsx-1 mRNA into the ventral blastomeres to see whether the native Xmsx-1 could cancel the dorsalizing effect of VP-Xmsx-1. As shown in Fig. I-3Ac, the dorsalized phenotype was almost completely rescued (0%, n=33). In contrast, ventral overexpression of eve-Xmsx-1 did not induce a secondary body axis (Fig. I-3Ae) and overexpression of eve-Xmsx-1 in dorsal blastomeres ventralized the embryo (Av.DAI 2.2, n=25)(Fig. I-3Ad), similar to the result with wild-type Xmsx-1 overexpression, showing that fusion of Xmsx-1 to the *eve* repressor domain retained the original activity of Xmsx-1. Taken together, these results show that forced transactivation of Xmsx target genes via the VP16 domain disrupted the normal function of endogenous Xmsx-1, and that Xmsx-1 may act as a transcriptional repressor in vivo.

To confirm the dorsalizing effect of VP-Xmsx-1 at the molecular level, particularly in mesoderm, I examined the expression level of mesodermal and other marker genes by RT-PCR (Fig. I-3B). Ventral mesoderm was excised from embryos ventrally overexpressing VP-Xmsx-1. Dorsal markers, *goosecoid*, *chordin* (Fig. I-3B) and *noggin* (data not shown), all of which mark Spemann's organizer (Cho et al., 1991; Sasai et al., 1994) were upregulated in the VMZ. *Xmyf-5*, which marks dorsolateral mesoderm (Hopwood et al., 1991) was also upregulated. On the other hand, the expression of the ventrolateral marker *Xwnt-8* (Christian and Moon, 1993) was attenuated to some extent, and the ventral marker *Xvent-1* was efficiently suppressed. Interestingly, all of the genes that were upregulated by VP-Xmsx-1 overexpression are normally suppressed in the

DMZ by Xmsx-1 or BMP overexpression, as shown in Fig. I-4. This result further supports the idea that Xmsx-1 is a transcriptional repressor and its target genes include organizer-specific genes such as *goosecoid* and *chordin*. Conversely, expression of *Xwnt-8* and *Xvent-1* appears to be increased by Xmsx-1 activity, because they are downregulated by overexpression of VP-Xmsx-1 and upregulated by wild type Xmsx-1. Given the nature of Xmsx-1 as a transcriptional repressor, Xmsx-1 may be repressing another repressor(s) acting on the upstream sequences of these genes.

VP-Xmsx-1 induces neural fate in ectoderm

Expression of Xmsx-1 mRNA is detected by RT-PCR in early embryonic development, starting at stage 4. *Xmsx-1* is highly expressed in the ectoderm (animal cap) of stage 8 1/2 embryos, as is BMP-4 (Suzuki et al., 1997b). The endogenous level of BMP-4 is thought to be necessary and sufficient to direct the ectoderm to differentiate into epidermis. Therefore, the dispersion of animal cap cells to remove the BMP-4 ligand, or the inhibition of BMP signal in animal cap cells by a dominant-negative BMP receptor leads to a loss of *Xmsx-1* expression (Suzuki et al., 1997b) as well as to the autonomous differentiation of the cells into neural tissues, as assessed by the expression of neural markers such as *Zic-3* and *NCAM* (Fig. I-5, lane 3). Based on this knowledge, I examined whether the loss of Xmsx-1 function in the animal cap would cause neural induction. VP-Xmsx-1 mRNA (250 pg) microinjected into the animal poles of 2-cell embryos induced *NCAM* and *Zic-3* expression (Fig. I-5, lane 2). The effect

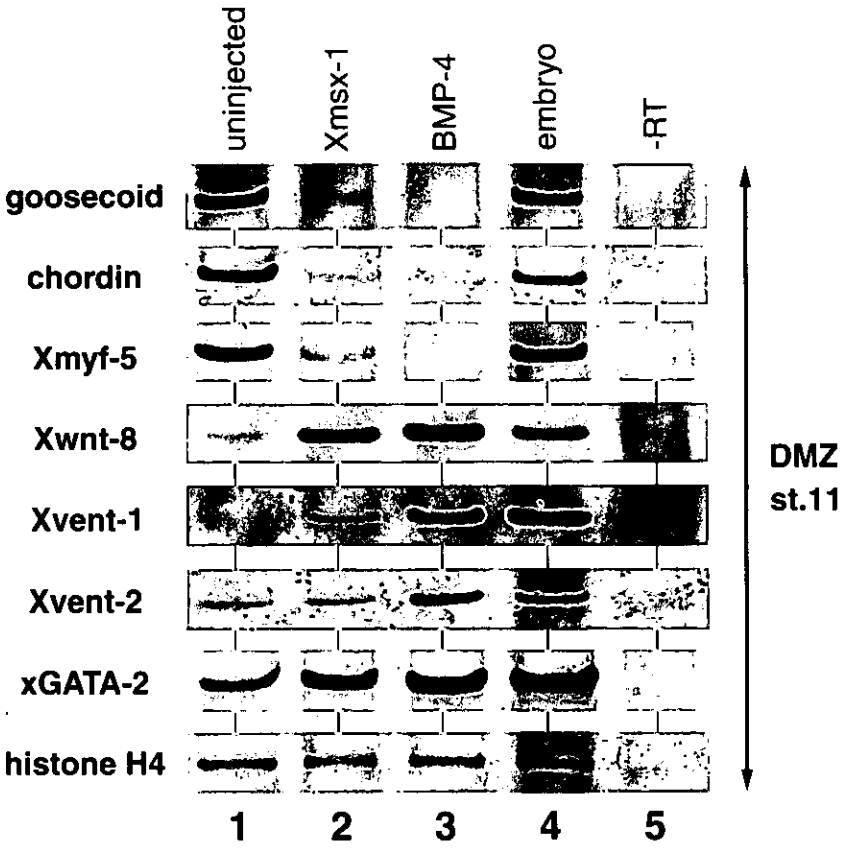


Fig. I-4. Reciprocal effect of wild-type Xmsx-1 and VP-Xmsx-1 on marker gene expression.
Embryos were either uninjected (lanes 1, 4, 5) or injected with 400 pg of Xmsx-1 (lane 2) or 200 pg of BMP-4 (lane 3) mRNA into the equatorial region of the two dorsal blastomeres at the 4-cell stage. Dorsal marginal zone explants (DMZ; lanes 1, 2, 3) were cultured until sibling embryos reached stage 11. Wild-type Xmsx-1 repressed and activated the expression of the genes activated and repressed by VP-Xmsx-1 (Fig. I-3B), respectively.

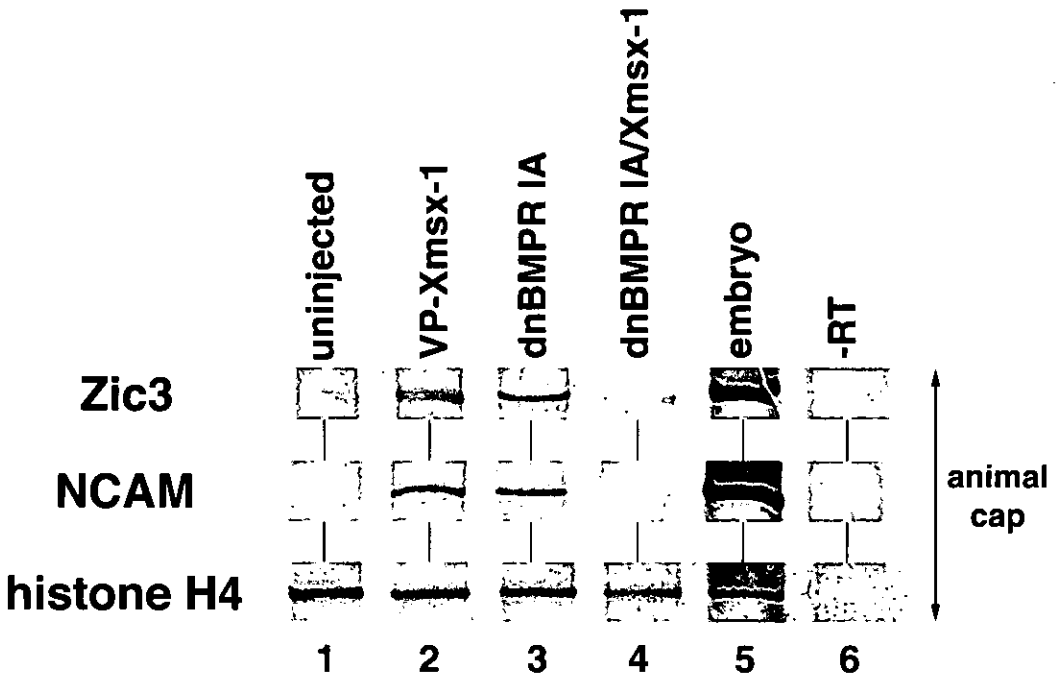


Fig. I-5. Neural induction by VP-Xmsx-1 expressed in ectoderm.
Animal poles of 2-cell stage embryos were either uninjected (lanes 1, 5, 6) or injected with 250 pg of VP-Xmsx-1 mRNA (lane 2), 200 pg of the truncated form of the BMP receptor (dnBMPR-IA) mRNA, which acts in a dominant-negative manner to inhibit the BMP signal (lane 3), or a mixture of 200 pg dnBMPR-IA and 1 ng Xmsx-1 mRNA (lane 4). Animal cap explants (lanes 1-4) were excised at the blastula stage (stage 8) and cultured until sibling embryos reached stage 11 (for *Zic3* expression) and stage 25 (for *NCAM* expression).

was similar to that caused by a dominant-negative BMP receptor (dnBMPR-IA), suggesting that VP-Xmsx-1 inhibited the signaling of BMP by blocking the function of Xmsx-1. This prediction was confirmed by the observation that coexpression of Xmsx-1 with dnBMPR-IA canceled the neural marker expression (Fig. I-5, lanes 3, 4), consistent with a previous observation (Suzuki et al., 1997b). Taken together, these findings indicate that Xmsx-1 is an essential mediator of BMP activity, and inhibition of Xmsx-1 activity is sufficient to induce neural fate in the ectoderm.

Xvent-1 acts downstream of Xmsx-1

It was previously reported that homeobox genes *Xvent-1* (Gawantka et al., 1995) and *Xvent-2* (Onichtchouk et al., 1996) are BMP-2/BMP-4 responsive genes. These genes are also expressed ventrolaterally and not in the dorsalmost region of early embryos, although the intensity of whole-mount in situ hybridization staining for these transcripts is relatively weak in ectoderm, compared with that for *Xmsx-1*. *Xvent-1* and *Xvent-2* were shown to mediate BMP activities, particularly in the ventralization of embryos in a manner reminiscent of *Xmsx-1* gene. The results in Figs I-3B and I-4 suggest that *Xvent-1* but not *Xvent-2* expression is dependent on the activity of Xmsx-1 because loss-of-function and gain-of-function manipulations of Xmsx-1 led to downregulation and upregulation of *Xvent-1* expression, respectively, despite the lack of effect on *Xvent-2* expression. To examine whether Xmsx-1 and Xvent-1 act in the same pathway, I analyzed the epistatic relationship between Xmsx-1 and Xvent-1. First, I tested whether dorsalization by ventral expression of VP-Xmsx-1

could be rescued by coexpressing of wild-type Xvent-1 or Xvent-2. As shown in Fig. I-6A, VP-Xmsx-1-induced dorsalized phenotypes (81% secondary axis, n=32)(Fig. I-6Aa) were successfully rescued by coexpression of Xvent-1 (84% normal, n=28)(Fig. I-6Ab), while the rescuing ability of Xvent-2 seemed to be lower than Xvent 1 (no secondary axis but Av.DAI 6.0, n=35). Marker gene expression also supported the idea that dorsalization by VP-Xmsx-1 was suppressed by Xvent-1 (data not shown). Moreover, overexpression of Xvent-1 in the dorsal marginal zone failed to induce *Xmsx-1*, while the ventrolateral marker *Xwnt-8* was induced and the dorsal markers *chordin* and *goosecoid* were completely suppressed (Fig. I-6B), confirming Xvent-1's ventralizing ability. To eliminate the possibility that the induction of *Xvent-1* by Xmsx-1 was caused by an increased level of BMP ligand, I examined the expression level of BMP in the DMZ of Xmsx-1-expressing embryos. There was no increase in BMP-2 or BMP-4 expression (data not shown). Taken together, these data suggest that Xvent-1 acts downstream but not upstream of Xmsx-1. To further confirm this sequence of events, I tested whether ventralization by Xvent-1 could be rescued by VP-Xmsx-1. If Xvent-1 acts downstream of Xmsx-1, the ventralized phenotype caused by increased levels of Xvent-1 activity should not be rescued by a dominant-negative mutant of the upstream gene *Xmsx-1*. As expected, the ventralized Xvent-1 phenotype (Av.DAI 2.0, n=29)(Fig. I-6Ac) was not rescued by VP-Xmsx-1 (Av.DAI 2.1, n=31)(Fig. I-6Ad). Conversely, ventralization by wild-type Xmsx-1 (Av.DAI 2.3, n=29)(Fig. I-6Ae) was partially rescued by VP-Xvent-1 (Av.DAI 4.0, n=31)(Fig. I-6Af), which was previously shown to interfere Xvent-1

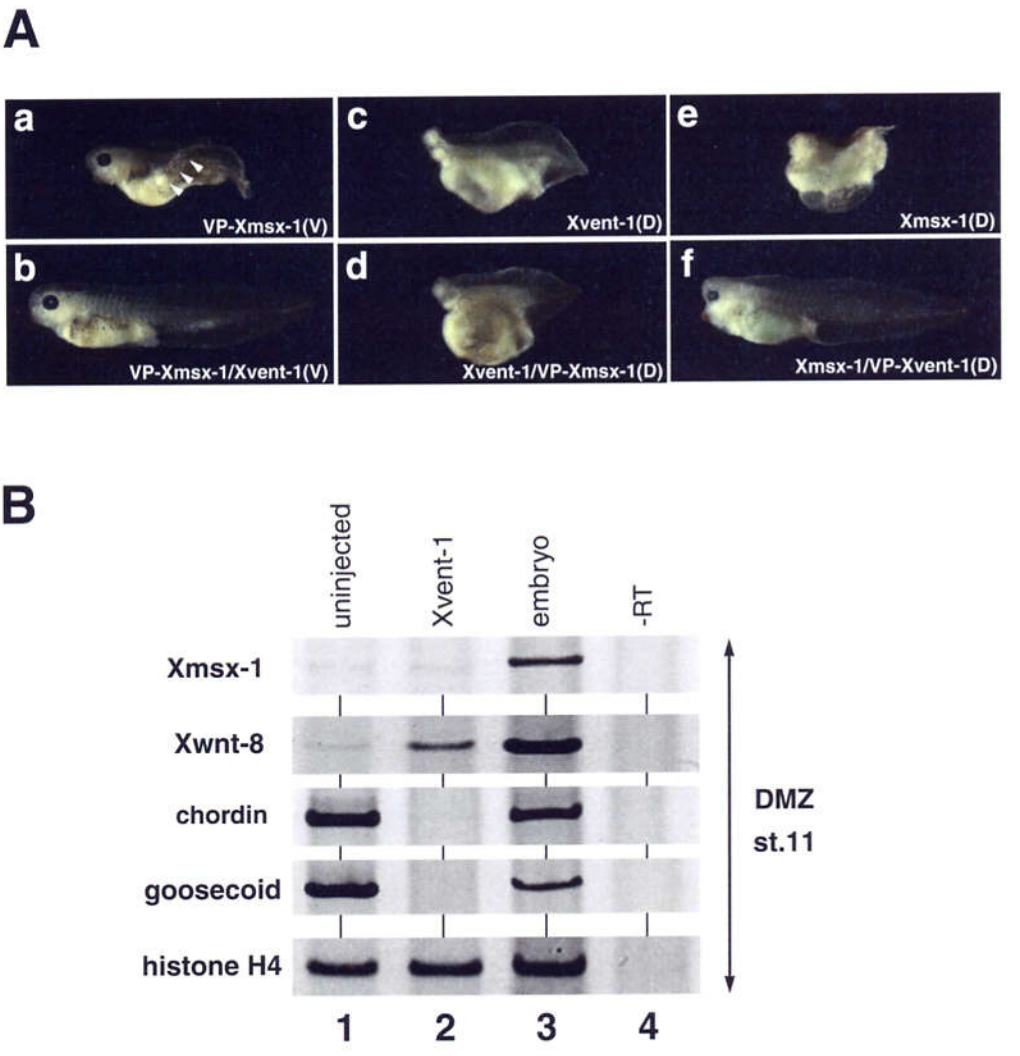


Fig. I-6. Xmsx-1 acts upstream of Xvent-1.
(A) 250 pg of VP-Xmsx-1 mRNA was either injected (a) or coinjected with 200 pg of Xvent-1 mRNA (b) into the ventral marginal region of 4-cell stage embryos. 400 pg of Xvent-1 mRNA was either injected (c) or coinjected with 200 pg of VP-Xmsx-1 mRNA (d) into the dorsal marginal region of 4-cell stage embryos. 500 pg of Xmsx-1 mRNA was either injected (e) or coinjected with 50 pg of VP-Xvent-1 mRNA (f) into the dorsal marginal region of 4-cell stage embryos. The white arrowheads indicate a secondary body axis. (B) Embryos were either uninjected (lanes 1, 3, 4) or injected with 500 pg of Xvent-1 mRNA (lane 2) into the dorsal equatorial region at the 4-cell stage. DMZs (lanes 1, 2) were excised at stage 10 and cultured until stage 11.

function and consequently to cause dorsalization of mesoderm and neural induction (Onichtchouk et al., 1998). Taken together, and given that *Xmsx-1* is an immediate early BMP-responsive gene, these results suggest that Xmsx-1 functions upstream of *Xvent-1*.

Xmsx-1 is required for early responses to ventralization by BMP

It is well established that BMP-4 is essential for the specification of both ventral mesoderm and ectoderm, and thus it seems likely that Xmsx-1 mediates the ventralizing activity of BMP as an essential component of the signaling pathway. Therefore, I next tested whether Xmsx-1 is required in the ventralizing pathway triggered by BMP. To test this possibility, I analyzed marker gene expression in the dorsal marginal zone where BMP-4 was overexpressed alone or with VP-Xmsx-1. The results clearly showed that ventralization of mesoderm by BMP-4 is efficiently canceled by the coexpression of VP-Xmsx-1 (Fig. I-7A). Expression of the dorsal marker *goosecoid*, which was eliminated by BMP-4 overexpression was completely restored by coexpressing VP-Xmsx-1. BMP-4-induced expression of the ventral markers *Xvent-1* and *Xwnt-8* was efficiently diminished by VP-Xmsx-1 overexpression. Given this fact, VP-Xmsx-1 should inhibit the ventralization of embryos induced by the BMP misexpression. I next evaluated the inhibiting activity of VP-Xmsx-1 against the ventralizing signal by the external appearance of tadpole stage embryos. UV-treatment of fertilized embryos inhibits the cortical rotation to decide dorso-ventral axis and consequently lead to misexpression BMP-4 in the dorsalmost region of gastrula (Fainsod et al., 1994) to ventralize the embryos. Thus,

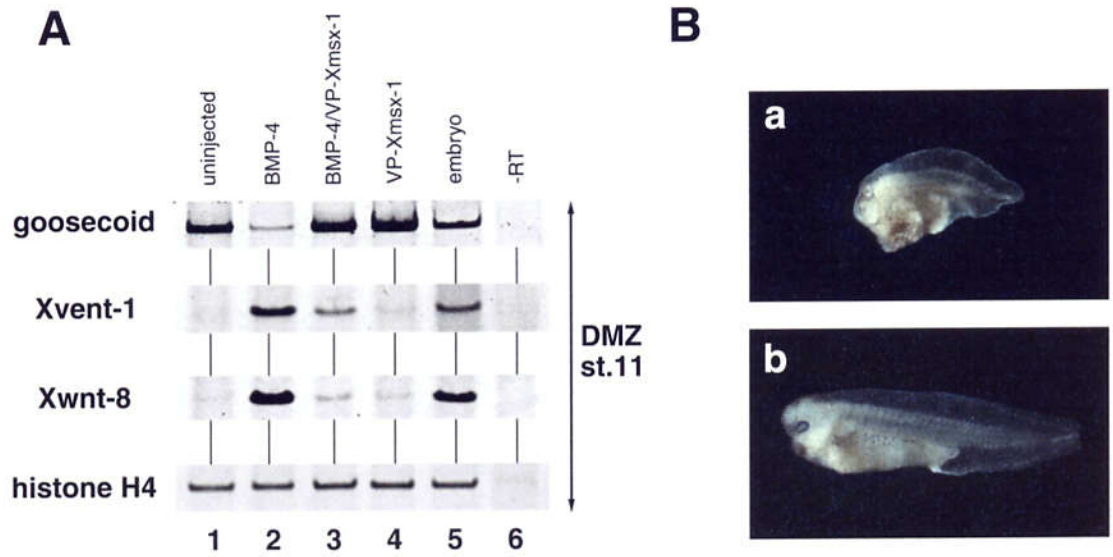


Fig. I-7. Xmsx-1 plays an essential role in the ventralization induced by BMP-4 signaling.
(A) VP-Xmsx-1 rescued the BMP-4-induced ventralization in gene expression of early stage embryos. Embryos were injected with 200 pg of BMP-4 mRNA (lane 2), 500 pg of VP-Xmsx-1 mRNA (lane 4), or a mixture of 200 pg BMP-4 and 500 pg VP-Xmsx-1 mRNA (lane 3) into the equatorial region of the two dorsal blastomeres at the 4-cell stage. DMZs were excised at stage 10 and cultured until stage 11. (B) VP-Xmsx-1 can rescue the ventralized phenotype caused by UV treatment assessed by external appearance. UV-treated embryo (a) was injected with 30 pg of VP-Xmsx-1 (b) into the equatorial region of the two blastomeres at the 4-cell stage. Phenotypes were evaluated at stage 38.

UV-treated embryos were injected with VP-Xmsx-1 mRNA. Ventralizing phenotypes by UV-treatment (Av.DAI 1.63, n=35)(Fig. I-7Ba) were partially rescued by expressing of VP-Xmsx-1 (Av.DAI 2.82, n=33)(Fig. I-7Bb). This result implies that VP-Xmsx-1 may inhibits BMP-4-induced ventralization. Given that the immediate early gene response of Xmsx-1 to the BMP signal occurs without protein synthesis, these results strongly support the idea that Xmsx-1 acts downstream of the BMP signal and is required for the ventralizing pathway triggered by BMP-4.

Discussion

Hierarchy of homeobox genes in the ventralizing pathway

Several homeobox genes, including *Xmsx-1, Xvent-1, and Xvent-2*, are known to be induced by BMP. In this study, I have shown by functional and induction analysis that there is an epistatic relationship between at least two ventralizing homeobox genes, *Xmsx-1* and *Xvent-1*, that is, Xmsx-1 acts upstream of Xvent-1. Given that Xmsx-1 is a transcriptional repressor, it is reasonable to speculate the existence of at least one more factor between Xmsx-1 and Xvent-1, that is responsible for repressing *Xvent-1* expression ("X" in Fig. I-8). One of the candidates for the repressor X is *goosecoid*, since the homeobox gene is expressed specifically in Spemann's organizer of the gastrula (Cho et al., 1991) and was shown to act as a transcriptional repressor (Artinger et al., 1997). In addition, the misexpression of *goosecoid* gene in the ventral marginal zone leads to the suppression of *Xvent-1* expression (Gawantka et al., 1995). Similar relationship has been reported between Xvent-2B and Xvent-1B which are members of *Xvent* homeobox gene family, showing that *Xvent-1B* is not a direct target of BMP-signaling and activated by Xvent-2B in expression level (Rastegar et al., 1999). It was previously reported that *Xmsx-1* and *Xvent-2* were immediate early BMP-responding genes (Ladher et al., 1996; Suzuki et al., 1997b). Furthermore, I have found that *Xvent-2* expression is neither induced in the DMZ by wild-type Xmsx-1 nor suppressed by VP-Xmsx-1 (Fig. I-6B). These results suggest that Xmsx-1 and Xvent-2 may be acting in independent pathways, although both are triggered by BMP (Fig.

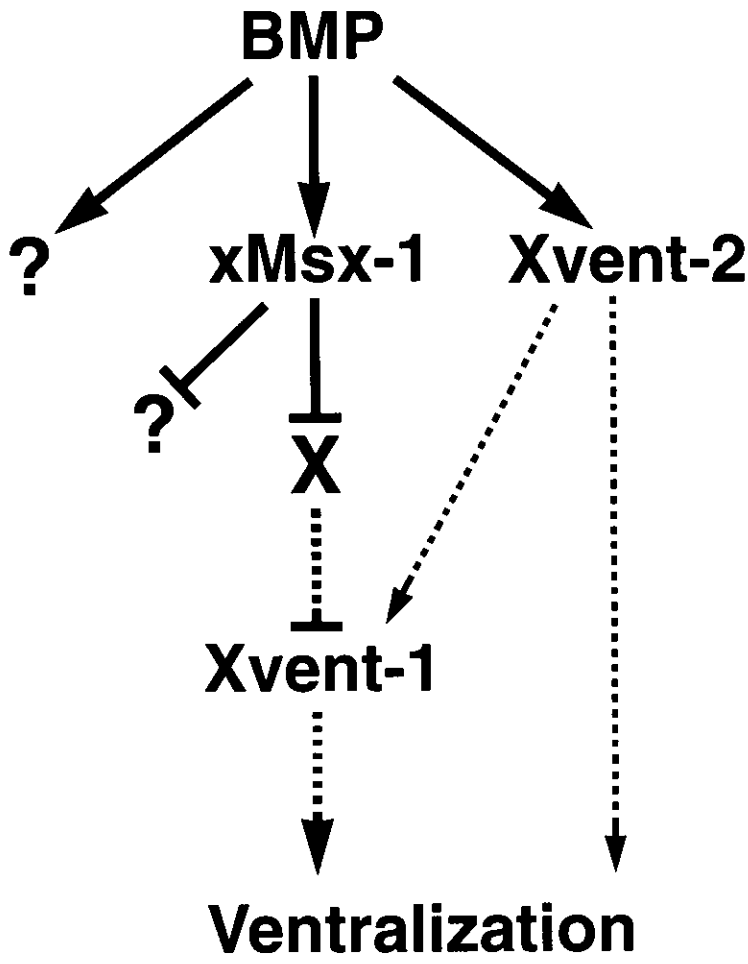


Fig. I-8. Model of the BMP-induced ventralizing signal in early development. Xmsx-1 induced by BMP signaling plays a major role in dorsoventral patterning in early development of *Xenopus laevis*. Xmsx-1 acts as an inducer of *Xvent-1*, the next ventralizing factor, by suppressing the transcription of a yet-unidentified transcriptional suppressor **X** for the *Xvent-1* gene, and consequently, the *Xmsx-1* expressing cells follow the ventral fate. The ventralizing signal from BMP and Xmsx-1, however, is also propagated to other redundant pathways including several other homeobox factors, Xvent-2 and Xmsx-2, and/or unknown factors (indicated by ?). Solid lines represent immediate early actions that occur without protein synthesis and broken lines represent the pathways that may be mediated by transcription of other factors.

I-8). The requirement for Xvent-2 in BMP-induced ventralization awaits further functional analyses.

Recently, xGATA-2, a member of the GATA family of zinc-finger transcription factors, which is expressed in the ventral mesoderm during gastrulation and required for hematopoietic differentiation (Kelley et al., 1993; Walmsley et al., 1994), has also been shown to induce *Xvent-1* but not to affect *Xvent-2* expression (Sykes et al., 1998). Furthermore, ventral overexpression of a dominant interfering GATA factor resulted in the formation of an incomplete ectopic axis, inducing *chordin* and *goosecoid* and repressing *Xwnt-8* (Sykes et al., 1998), much like VP-Xmsx-1. Interestingly, *xGATA-2* expression was upregulated by overexpression of Xmsx-1 in the DMZ (Fig. I-4) and effectively downregulated by VP-Xmsx-1 in the VMZ (Fig. I-3B) suggesting that xGATA-2 acts downstream of Xmsx-1. However, unlike Xmsx-1, GATA factors neither affect ectodermal cell fate directly nor ventralize the mesoderm when expressed in their native form (Sykes et al., 1998). Taken together, these results suggest that Xmsx-1 and GATA factors might share similar properties and function through a partially overlapping pathway to regulate mesoderm formation.

Does Xmsx-1 represent all BMP activities?

It is well understood that there are several homeobox genes that can mimic BMP activities, which include epidermal induction and neural inhibition. Therefore, I predict that there are redundant ventralizing pathways even within the BMP-regulated pathway. On the other hand, an attempt to rescue the dnBMPR-IA-induced dorsalized embryo by

coexpressing wild-type Xmsx-1 demonstrated that Xmsx-1 is sufficient to rescue the secondary body axis and restore the blocked BMP signal (data not shown). In the animal cap, Xmsx-1 also reversed the *NCAM* induction and epidermal keratin suppression caused by dnBMPR-IA (Suzuki et al., 1994). These results support the previous idea that Xmsx-1 can mediate all the ventralizing activities of BMP. In this study, I attempted to perform the reverse experiment by blocking BMP-induced ventralization with VP-Xmsx-1. In fact, VP-Xmsx-1 reversed the BMP-induced marker gene expression in the DMZ: the suppression of *goosecoid* and the increased levels of *Xvent-1* and *Xwnt-8* expression were returned to normal (Fig. I-7A). This result again supports the idea that Xmsx-1 mediates all testable activities of BMP. Moreover, I also showed that ventralizing phenotypes by UV-treatment were partially rescued by dorsally expressing of VP-Xmsx-1 (Fig. I-7B). This suggests that Xmsx-1 normally mediates endogenous ventralizing pathways, including BMP. However, when BMP-4 mRNA was injected dorsally alone or with VP-Xmsx-1 mRNA, the ventralization triggered by BMP-4 mRNA injection was not inhibited by coexpressing VP-Xmsx-1, evaluating the phenotypes of tadpole stage embryo (data not shown). In fact, the ventralized phenotype appeared to be even enhanced. There are possible explanations for this puzzling observation. One possibility is that the half-life of the VP-Xmsx-1 protein is limited and that its effect lasts only for a shorter period after translation than BMP-4. Another is that a redundant ventralizing pathway(s) is overactivated during the development of BMP-4- and VP-Xmsx-1-coinjected embryos, upon the inhibition of Xmsx-1 activity. Since marker gene expression was

examined when sibling embryos reached stage 11, but the phenotype of the ventralized embryos was observed at stage 38, it is possible that such compensation occurred during that time. To test this possibility, I examined whether expression of other ventralizing genes was upregulated upon overexpression of VP-Xmsx-1. Although I examined the expression level of *Xvent-2* and *Smad-1* in the DMZ after the injection of VP-Xmsx-1, their expression levels were unchanged (data not shown), but the possibility remains that such a compensation mechanism exists for other ventralizing genes. The unrescued phenotype at tadpole stage also suggests that dorsalization by VP-Xmsx-1 did not occur solely depending on the induction of *chordin* and *noggin* though they are induced by VP-Xmsx-1, because if the dorsalization depends on them, BMP effects should be cancelled by VP-Xmsx-1 even at the late stage.

Xmsx-1 is a suppressor of organizer-specific genes

Recently, Laurent and Cho (1999) proposed a model whereby BMP activity ventralizes the area expressing BMP itself by interfering with the induction of organizer-specific genes such as *goosecoid*, which is stimulated by activin, Vg1, and nodal. *goosecoid* is induced by two homeobox genes Xtwn and Xsia regulated by the Wnt pathway. Laurent and Cho (1999) have shown that BMP signaling can antagonize the induction of *goosecoid* without affecting Wnt signaling at the level of Xtwn. These results suggest that the antagonistic activities of BMPs in organizer formation occur independently of twin regulation. My present study has demonstrated that Xmsx-1 has a potent antagonistic activity in

organizer formation, which includes suppression of the organizer specific genes *goosecoid* and *chordin*. It is most likely that Xmsx-1 plays a major role in mediating the anti-organizer activity of BMP. In other words, BMP may antagonize organizer activity through Xmsx-1. Expression of *goosecoid* is regulated by the combination of two homeobox binding elements, distal element (DE) and proximal element (PE) (Watabe et al., 1995). It is tempting to speculate that Xmsx-1 might mediate interference with the homeobox binding elements in the promoter region of *goosecoid*, directly or indirectly. This may lead to an interesting model regarding how the organizer is established, as organizer formation may require cancellation of the negative regulation of *goosecoid* expression by Xmsx-1. Specifically, there may be antagonism between Xmsx-1 and the *goosecoid*-activating homeobox proteins Xtwn and Xsia. All of these hypotheses need to be tested in the near future.

PART II

Suppression of head formation by Xmsx-1 through the inhibition of intracellular nodal signaling

Summary

It is well established that in *Xenopus*, BMP ventralizes the early embryo through the activation of several target genes encoding homeobox proteins, some of which are known to be necessary and sufficient for ventralization. Here, I used an inhibitory form of Xmsx-1, one of BMP's targets, to examine its role in head formation. Interestingly, ventral overexpression of a dominant Xmsx-1 inhibitor induced an ectopic head with eyes and a cement gland in the ventral side of the embryo, suggesting that Xmsx-1 is normally required to suppress head formation in the ventral side. Supporting this observation, I also found that wild-type Xmsx-1 suppresses head formation through the inhibition of nodal signaling, which is known to induce head organizer genes such as *cerberus*, *Xhex*, and *Xdkk-1*. I propose that negative regulation of the BMP/Xmsx-1 signal is involved not only in neural induction but also in head induction and formation. I further suggest that the inhibition of nodal signaling by Xmsx-1 may occur intracellularly, through the interaction with Smads, at the level of the transcriptional complex activating activin responsive element.

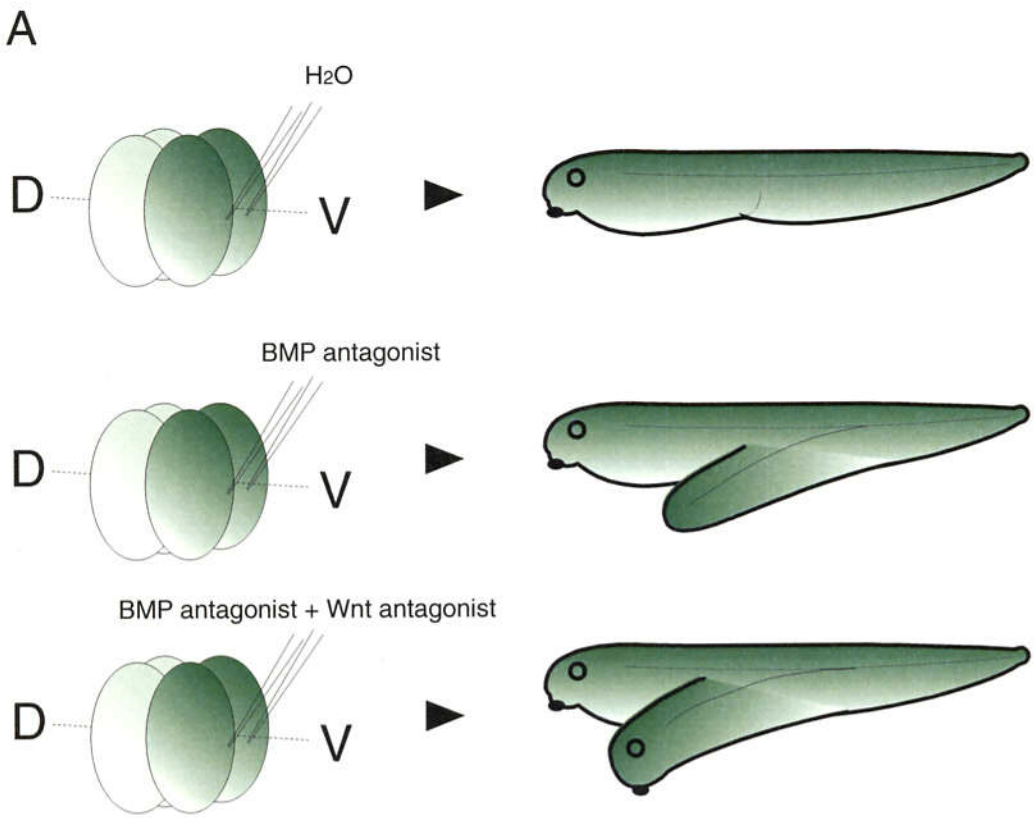
Introduction

The establishment of the dorsoventral axis and subsequent pattern formation along the axis are known to be regulated by several polypeptide growth factors (PGFs) belonging to the TGF-β family (Beddington and Robertson, 1999; Heasman, 1997). In the last decade, two distinct roles for these family members have been clarified: the dorsal fate is determined by the activin/nodal/Vg1 class of ligands and the ventral fate by the BMP class of ligands (Hogan, 1996). Thus, current efforts are focused on elucidating the molecular mechanisms by which tissues are differentially induced by these signals. In particular, nodal has been shown in both zebrafish and mouse to play essential roles in endoderm and mesoderm differentiation and the subsequent cell movement during gastrulation (Conlon et al., 1994; Feldman et al., 1998; Sampath et al., 1998; Varlet et al., 1997). Recently, a rapid accumulation of knowledge from zebrafish genetics and other studies has helped clarify the role of nodal signaling in establishing left-right asymmetry. In contrast, BMPs are essential for specifying both the ventral mesoderm and cell fate in the non-neural ectoderm (Sasai and De Robertis, 1997; Weinstein and Hemmati-Brivanlou, 1999; Wilson and Hemmati-Brivanlou, 1995).It has been shown that in the absence of BMP activity caused by the BMP antagonists noggin, chordin, and follistatin, which are secreted from Spemann's organizer, the presumptive ectoderm differentiates into neuronal cells by inhibiting the epidermal fate (Iemura et al., 1998; Piccolo et al., 1996; Sasai

et al., 1995; Weinstein and Hemmati-Brivanlou, 1999; Zimmerman et al., 1996). Studies of the signal transduction mechanism of BMP family ligands have identified several target genes that are necessary and sufficient for BMP signaling. Some of these are immediate early genes that respond to BMP signals without de novo protein synthesis (Ladher et al., 1996; Miyama et al., 1999; Suzuki et al., 1997b). Most of the BMP target genes identified to date encode homeobox proteins, including Xmsx-1 (Suzuki et al., 1997b), Xmsx-2, Xvent-1 (Gawantka et al., 1995), Xvent-2 (Onichtchouk et al., 1996), and Xdll-3 (Dlx5) (Miyama et al., 1999).

Ectopic activation of the activin/nodal/Vg1 signal or inhibition of the BMP signal induces a partial dorsal axis (Fagotto et al., 1997; Lustig et al., 1996; Suzuki et al., 1994; Thomsen et al., 1990), suggesting that the actions of these two classes of growth factors are reciprocal. The induction of a partial secondary axis is considered as a consequence of their intracellular signal transduction and target gene activation. Thus, this experimental system has been useful for studying the in vivo molecular mechanisms of dorsalization and ventralization in the early *Xenopus* embryo. In contrast, head formation has been thought of as a related but distinct molecular event from trunk formation, in which only TGF-β family ligands are involved (Niehrs, 1999). The role of BMP-2 and BMP-4 has been believed to be limited to suppressing the trunk, because inhibiting them results in a partial dorsal axis with no head (Suzuki et al., 1994). Thus, the complete dorsal body axis has been believed to be patterned by two independent activities the "head organizer" and the "trunk organizer". In contrast to the action of BMP as a trunk repressor,

the Wnt family of proteins has long been implicated in head formation. One of the Wnt family members, Xwnt-8, was initially reported to induce an ectopic secondary dorsal axis with a complete head when its mRNA was injected into ventral blastomeres (Christian et al., 1991). It was later found, however, that *Xwnt-8* is normally expressed in the ventrolateral mesoderm during the early gastrula stage and that dorsal overexpression by DNA injection ventralized the embryo, suggesting that endogenous Xwnt-8 acts as a ventralizing agent (Christian and Moon, 1993). This interpretation implied that the Wnt ligand is involved in head repression rather than head induction. Recent studies have further demonstrated that in addition to inhibition of the BMP signal, inhibition of the Wnt signal is necessary for head induction, designated Two-inhibitor model (Fig. II-1), suggesting that the default, inhibited, state of both of these signals is required for head induction (Glinka et al., 1997). Recently however, cerberus, a multiple binding protein for Wnt, nodal, and BMP, was reported to be responsible for head induction (Piccolo et al., 1999). Because the late overexpression of nodal by DNA injection into the dorsal side causes repression of the head, inhibition of nodal by cerberus may be essential for head formation. In addition, a more recent study showed that the inhibition of Wnt ligands in ventral blastomeres by ECD8, an extracellular domain of the Wnt receptor Frizzled, is sufficient to induce an ectopic head (Itoh and Sokol, 1999). Therefore, the precise sequence of gene activation and protein-protein interaction events that promote head development in vivo is still unclear.



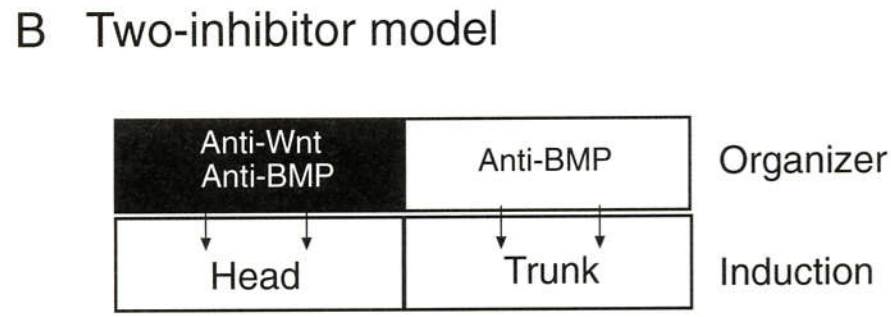


Fig. II-1. Head formation by anti-BMP and anti-Wnt activity.
(A) Experiments for ectopic head formation. Ectopic overexpression of an anti-BMP activity by microinjecting with synthetic mRNA for BMP antagonist results in a secondary trunk lacking head. But a complete secondary axis including head structure is induced by coinjecting with mRNAs for BMP antagonist and Wnt antagonist. (B) Model for region-specific anterioposterior induction by the Spemann's organizer (Two-inhibitor model). Trunk induction requires inhibition of BMP signal and head induction requires inhibition of both BMP and Wnt signal.

Although many of the experiments on head formation in *Xenopus* have involved looking at the effects of extracellular ligands, in this study, I approached the problem using an inhibitory version of the homeobox protein Xmsx-1. Here I show that Xmsx-1 has an essential role in head repression that is elicited at the level of the transcriptional complex induced by nodal signaling.

Experimental procedures

Constructs

Activating forms of Xmsx-1 were created by replacing the N-terminal 136 aa (TI-Xmsx-1) or the first methionine (HI-Xmsx-1) of Xmsx-1 with the 81-aa activation domain of VP16 protein (VP16 AD). Xsmad1, 4α, and 4β cDNAs were isolated by polymerase chain reaction (PCR) from a cDNA library synthesized from stage 10 embryos. A Flag epitope (DYKDDDDK) was N-terminally inserted between first Met and Ala2 of Xmsx-1 for the immunoprecipitation experiment. An HA epitope (YPYDVPDYA) was inserted into the N-terminus of Xsmad1 and 2 and the C-terminus of Xsmad4α and 4β for Western blot analysis. The Xsmad4β coding sequence was ligated into the EcoRI site of pGEX-4T-3 to produce the Xsmad4β-glutathione S-transferase(GST) fusion protein for GST pull-down analysis. The pCS2VP16 plasmid, which contains the VP16 activation domain, and the -226gsc Luc construct were gifts from Dr. Ken W. Y. Cho. The Xsmad2 cDNA and GST-xFAST-1 construct were gifts from Drs. Douglas A. Melton and Malcolm Whitman, respectively.

Manipulation of embryos and microinjection of synthetic mRNA

In vitro fertilization of *Xenopus* eggs was performed as described previously (Suzuki et al., 1995). The fertilized embryos were dejellied using 3% cysteine hydrochloride and washed with water several times. Four-cell-stage embryos were microinjected with capped mRNAs, which

were synthesized using the mMESSAGE mMACHINE sp6 kit (Ambion) and then purified with Sephadex G-50 column (Amersham Pharmacia Biotech). The injected embryos were cultured in 3% Ficoll/0.1x Steinberg's solution until the appropriate stage for each experiment. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). The VMZ, DMZ, ventral vegetal quarter, and dorsal vegetal quarter were dissected at stage 10 and cultured in 0.1% bovine serum albumin (BSA)/1x Steinberg's solution (Asashima et al., 1990).

RT-PCR analysis

Total RNA was extracted from the marginal zone explants and the embryos using TRIzol reagent (GIBCO/BRL) according to the manufacturer's instructions. Extracted RNA was subjected to reverse transcription with random hexameric primers. The expression of each molecular marker was detected by PCR using the following specific primers: anterior endomesodermal markers, *cerberus*, upstream, 5'-ATTCACTTAACAGCAGAGGT-3' and downstream, 5'-CTTCTAGAACCATTGTAAGC-3'; *Xotx-2*, upstream, 5'-GGAGGCCAAAACAAAGTG-3' and downstream, 5'-TCATGGGGTAGGACCTCT-3'; *Xhex*, upstream, 5'-AAGAGCCAAATGGAGGCGTC-3' and downstream, 5'-GCAAGCTGAATAGAGGTCCA-3'; *Xdkk-1*, upstream, 5'-CTCTACAGTTGCACGGAAGA-3' and downstream, 5'-CCAGAATGGTTTCTTCCAGG-3'; a pan mesodermal marker, *Xbra*, upstream, 5'-GGATCATCTTCTCAGCGCTGTGGA-3' and downstream, 5'-

GTTGTCGGCTGCCACAAAGTCCA-3'; nodal related gene markers, *Xnr-1*, upstream, 5'-AACCTCCCAAGCCTACTGGA-3' and downstream, 5'-TTGTGTGATGGTTCAGTCTC-3'; *Xnr-2*, upstream, 5'-GTCTTCTATATCCAGCAGCAAT-3' and downstream, 5'-TTGATGGAGATAATACTGGAGC-3'; *Xnr-3*, upstream, 5'-GCCTCCCTTCTTTTAGAAAG-3' and downstream, 5'-CATCGTATCTACATTTTCTG-3'; *Xnr-4*, upstream, 5'-ACTTGGCTGCTCTACCTC-3' and downstream, 5'-CAGCAAGTTGATGTTCTTCC-3'; *HoxB9*, upstream, 5'-TACTTACGGGCTTGGCTGGA-3' and downstream, 5'-AGCGTGTAACCAGTTGGCTG-3'; *eFGF*, upstream, 5'-TTACTGCAATGTGGGCATCG-3' and downstream, 5'-GCAGAAGCGTCTCTTTGAAT-3'. The primer sequences for *Xwnt-8*, *Xvent-1*, *Xvent-2*, ventrolateral markers, and *histone H4*, an internal input control, were as described in Part I.

Whole-mount in situ hybridization, immunostaining, and lineage tracing

Embryos coinjected with HI-Xmsx-1 and β-galactosidase mRNA were fixed in 4% paraformaldehyde in 0.1 M potassium phosphate buffer (pH 7.8), washed with PBS, and stained with 6-chloro-3-indolyl-β-D-galactoside (nacalai tesque) for lineage labeling. Stained embryos were then refixed in MEMFA [0.1 M MOPS (pH7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% Formaldehyde] and stored in methanol at -30°C before being used for whole-mount in situ hybridization. Whole-mount in situ hybridization

was performed as described previously (Harland, 1991; Hemmati-Brivanlou et al., 1990) using BM Purple (Boehringer) for the color reaction. To visualize the staining in the endomesoderm the embryos were cut with a razor blade along the dorsoventral axis.

Whole-mount immunostaining with anti-phosphoSmad1 antibody (Faure et al., 2000) and anti-MSX-1 (Babco) were performed as follows: embryos were fixed in MEMFA for 1 hour at room temperature, then washed with PBS and stored in methanol at -30°C before being used. The fixed embryos were rehydrated in PBS, permeabilized in PBSTw (0.1% Tween 20 in PBS), blocked in 15% fetal bovine serum and incubated in primary antibody solution (1/500 and 1/2,000 dilution for anti-phosphoSmad1 and anti-MSX-1 antibody, respectively) overnight at 4°C. After washing with PBSTw several times, the secondary antibody incubation with HRP-conjugated goat anti-rabbit IgG (1/1,000 dilution) was carried out for 1 hour at room temperature and followed by washes with PBSTw. Immunoreactivity was visualized with diaminobenzidine. Immunostaining with the monoclonal antibody MZ15 was performed as described (Klymkowsky and Hanken, 1991).

Luciferase reporter assay

Four-cell-stage embryos were injected marginally with 50 pg of the -226gsc/Luc (Watabe et al., 1995) construct alone or in combination with the indicated capped synthesized mRNAs as described above. At stage 10.25, or 10.5, five DMZs or VMZs were isolated and lysed with 30 µl of cell lysis

buffer [25 mM Tris-phosphate (pH 7.8), 2 mM Dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100]. For the luciferase assay, 100 µl of Luciferase Assay Substrate (Promega) were added to each extract and luciferase activity was measured using a Luminescencer-PSN AB-2200 (ATTO).

Immunoprecipitation and GST pull-down analysis

293T cells were transiently transfected with the indicated constructs by the calcium phosphate method. Forty hours after transfection, the cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF] in the presence of protease inhibitors. Immunoprecipitation and the GST pull-down analysis were performed by incubating the extracts with the M2 Flag monoclonal antibody (Sigma) coupled to protein A Sepharose CL 4B and with Glutathione Sepharose 4B (Pharmacia), respectively, at 4°C for 1 hour. The precipitates were then washed with lysis buffer and subjected to Western blot analysis using an anti-HA antibody (SANTA CRUZ BIOTECHNOLOGY).

Results

Xmsx-1 suppresses head formation

Xmsx-1 is expressed in the ventral ectoderm and mesoderm of early *Xenopus* embryos, suggesting a role for it as a mediator of BMP-4 signaling (Suzuki et al., 1997b). In addition, Xmsx-1 activity is required downstream of BMP for the ventralization of the mesoderm and ectoderm as shown in Part I. Dorsal overexpression of Xmsx-1 by mRNA injection (0.25 ng of mRNA) caused a headless embryo with no significant defects in trunk formation (40.0%, n=35; Fig. II-2B), as reported previously (Suzuki et al., 1997b). This phenotype is similar to that caused by the dorsal overexpression of BMP-2, BMP-4 (Dale et al., 1992; Jones et al., 1992), or BMP-7 (Iemura et al., 1998). The failure of head formation by Xmsx-1 as well as by BMP-4 was found to be tightly correlated with a marked reduction in the anterior endomesoderm markers *Xotx-2*, *Xhex*, and *Xdkk-1*, all of which define head organizer activity (Fig. II-2C). These results suggest that Xmsx-1, which is normally expressed in the ventral side of the embryo, may play a role in the negative regulation of head formation. Thus, the following experiments were undertaken to examine whether such suppression of head formation and release from negative regulation are part of the mechanism underlying normal head development in *Xenopus*.

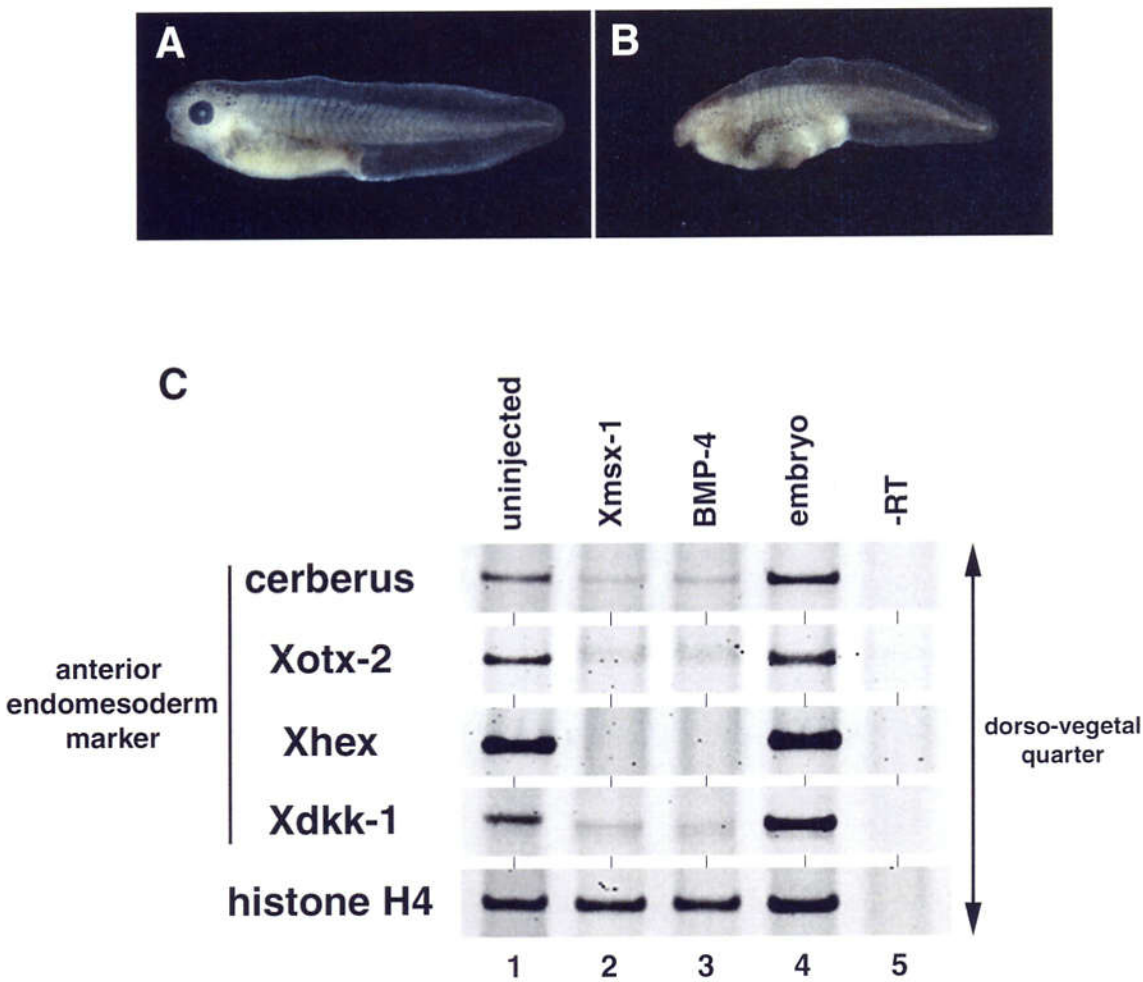


Fig. II-2. Ectopic expression of Xmsx-1 in the anterior endoderm suppresses head structure formation.

Xenopus embryos were either uninjected (A, C lanes 1, 4, 5) or injected with 500 pg of Xmsx-1 (B, C lane 2) or 200 pg of BMP-4 (C lane 3) mRNA in the submarginal zone of the two dorsal blastomeres at the 4-cell stage. (A, B) The embryos were cultured until stage 40 to observe the phenotypes. (C) RT-PCR analysis for the early anterior endomesodermal markers. The dorso-vegetal quarters of the embryos were dissected at stage 10.25 (lanes 1-3) and then subjected to RT-PCR analysis for the indicated markers. Lanes 4 and 5, whole embryo control with (lane 4) or without (lane 5) the RT step.

An inhibitory form of Xmsx-1 induces an ectopic head

To examine the requirement of Xmsx-1 in *Xenopus* embryogenesis, I have constructed cDNAs encoding fusion proteins of Xmsx-1 with either the VP16 transcriptional activation domain or an even-skipped-derived transcriptional repressor domain in Part I. I used these dominant-inhibitory forms to show that Xmsx-1 is an essential component of the BMP pathway and that it is involved in the ventral specification of both the mesoderm and ectoderm. Later, I found that another version of Xmsx-1 induces ectopic head when ventrally overexpressed. This newly constructed fusion protein, VP16-full-Xmsx-1, in which the VP16 activation domain is added to the N-terminus of the full-length protein is shown in Fig. II-3A, middle, and can be compared with the previously reported protein, VP16-Xmsx-1 (Fig. II-3A, bottom). Although Xmsx-1 was originally found to be a transcriptional repressor for some of the BMP-target genes, such as *Xvent-1*, both fusion proteins inhibit the ventralizing action of wild-type Xmsx-1 when overexpressed in dorsal blastomeres. In particular, I found that VP16-full-Xmsx-1 acts as an even more potent inhibitor of Xmsx-1 and efficiently rescues the Xmsx-1-induced ventralized phenotype at lower doses of mRNA than did VP16-Xmsx-1 (data not shown). The specificities of the ability of both of the inhibitory forms to rescue the ventralized phenotype were indistinguishable, and they had no inhibitory activity against another ventralizing homeobox genes, *Xdll-3*, but they weakly inhibited *Xmsx-2*, a homeobox gene that is closely related to *Xmsx-1*, as described in Part I (data not shown).

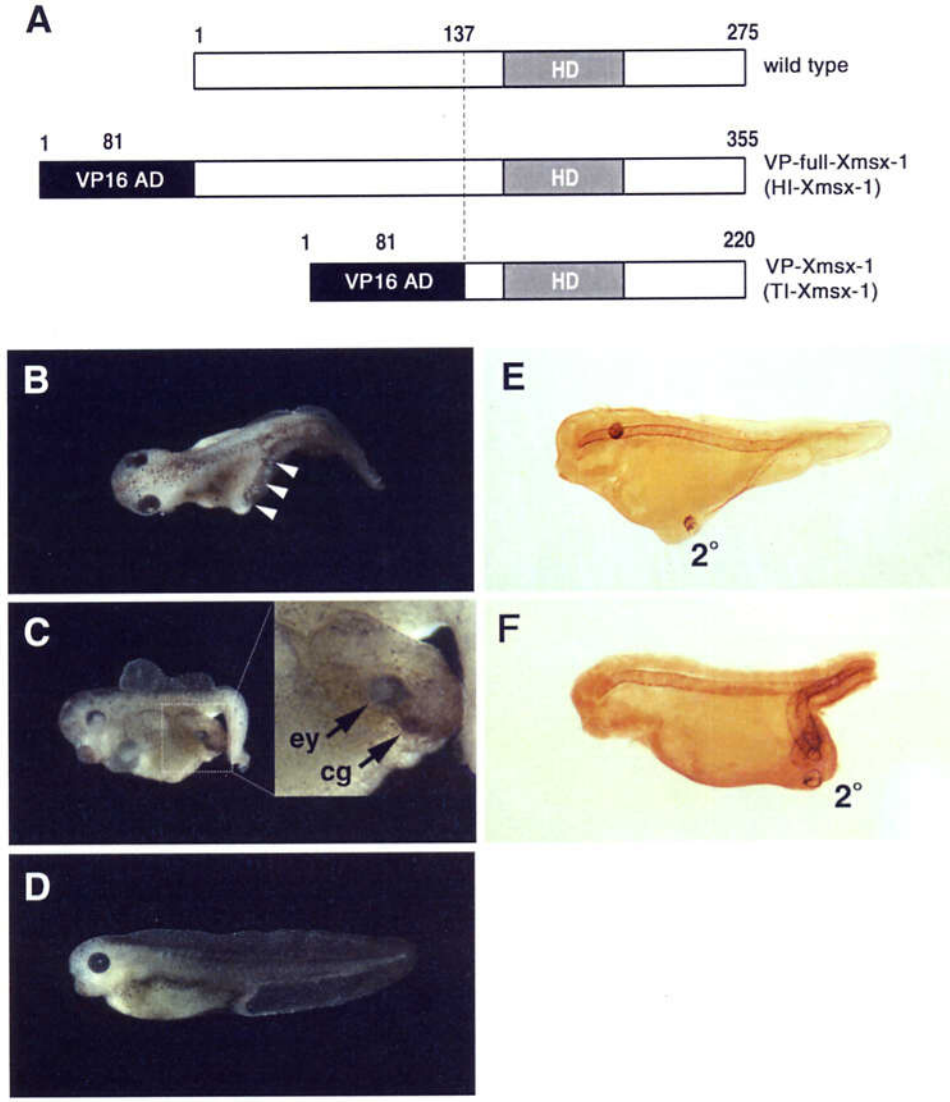


Fig. II-3. Ventral overexpression of HI-Xmsx-1 induces an ectopic head.
(A) Schematic drawing of the Xmsx-1 activating forms. (top) Wild-type Xmsx-1 protein. Activating forms of Xmsx-1, HI-Xmsx-1 (middle), and TI-Xmsx-1 (bottom), were constructed by fusing the 81 amino acids of the VP16 activation domain (black box, VP16 AD) with the full-length and C-terminal 137 amino acids' region of Xmsx-1 protein, respectively. The amino acid numbers of several junction sites are shown above each drawing. The homeodomain is indicated by the gray box (HD). (B, C, D) Phenotypes of mRNA-injected embryos. (E, F) Staining with the notochord- and otic vesicle-specific antibody, MZ15. Four-cell stage *Xenopus* embryos were ventrally injected with 500 pg of TI-Xmsx-1 (B, E) or HI-Xmsx-1 (C, F) mRNA alone or coinjected with 500 pg of HI-Xmsx-1 and 1 ng of wild-type Xmsx-1 mRNA (D). HI-Xmsx-1-injected embryos formed a secondary axis with an ectopic head containing an eye, cement gland and short notochords in the ventral side. Ectopic head region is magnified in the right side of panel B. The ectopic head phenotype was rescued by wild-type Xmsx-1. An ectopic trunk axis is indicated by the white arrowheads. cg, cement gland; ey, eye; 2, secondary axis.

More interestingly, when 500 pg of VP16-full-Xmsx-1 mRNA was overexpressed in the ventral two blastomeres of a 4-cell-stage embryo, an ectopic head was induced (Fig. II-3C), but the same or the increasing dose of VP16-Xmsx-1 mRNA never induced head but a partial secondary axis (Fig. II-3B). Thus, I redesignated the new (VP16-full-Xmsx-1) and former (VP16-Xmsx-1) Xmsx-1 fusion proteins, both of which act in a dominant inhibitory way, as the "head-inducing" Xmsx-1 (HI-Xmsx-1) and "trunk-inducing" Xmsx-1 (TI-Xmsx-1), respectively. As shown in Fig. II-3C, ventrally injected HI-Xmsx-1 induced a rather short secondary axis with an ectopic head structure that included eyes and a cement gland, which represent the most anterior tissues (Table II-1). Significant truncation of the primary axis, particularly in the tail region, was also observed. Surprisingly, staining with the notochord- and otic vesicle-specific antibody, MZ15, showed the presence of the notochord structure in the HI-Xmsx-1-induced short secondary axis (73%, n=11), while no notochord was induced with TI-Xmsx-1 (0%, n=10) (Fig. II-3E,F). This anteriorizing phenotype was rescued by coinjecting with wild-type Xmsx-1 (Fig. II-3D) (Table II-1).

To understand the molecular basis of the ectopic head induction, I performed RT-PCR analysis to detect either induced or suppressed genes (Fig. II-4A). I found that the so-called head organizer genes, *Xotx-2*, *cerberus*, *Xhex*, and *Xdkk-1*, which are normally expressed in the anterior endomesoderm and are essential for head formation, were ectopically induced at high levels in the ventral endomesodermal zone of the HI-Xmsx-1-expressing embryos (Fig. II-4A,B), suggesting that Xmsx-1 is

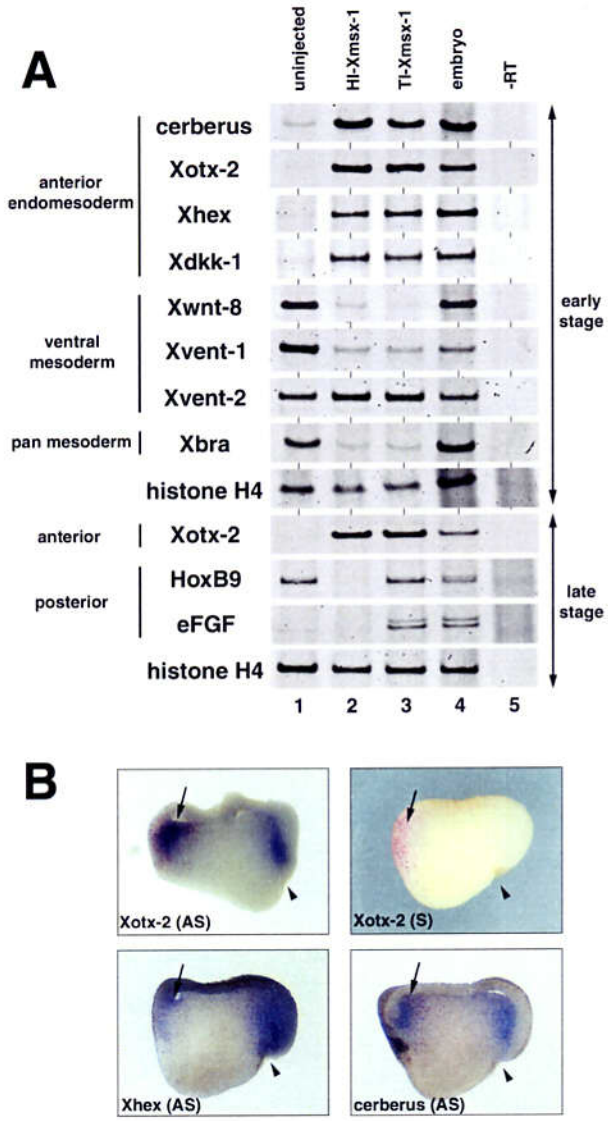


Fig. II-4. HI-Xmsx-1 induces head organizer gene markers in the ventral endomesoderm.

Two ventral blastomeres of 4-cell-stage embryos were coinjected marginally with 100 pg of HI- or TI-Xmsx-1 mRNA and 50 pg of β-galactosidase mRNA as a lineage tracer, then processed for RT-PCR (A) and whole-mount in situ hybridization (B) at stage 10.25. (A) The ventro-vegetal quarter of the embryos injected with the indicated mRNA (top, lanes 1, 2, 3) was dissected at stage 10.25 and RNA was extracted immediately for early stage or after cultured until sibling embryos reached stage 18 for late stage. RNA extracted from each explant was analyzed by RT-PCR. Lane 4 shows the expression of each marker in whole embryos and lane 5 shows the control reactions with no RT step. (B) The injected embryos were stained by red Gal for lineage tracing and then analyzed by whole-mount in situ hybridization for *Xotx-2*, *Xhex*, and *cerberus* gene expression. Anterior organizer genes were induced in the ventral endomesoderm. The dorsal blastopore lip is indicated by the black arrowhead. Arrow shows the RNA-injected ventral marginal zone, in which the nuclei of cells are stained red. AS, antisense probe; S, sense probe.

normally required in the ventral endomesoderm to repress head organizer gene expression. In addition, it was noted that expression of the ventral mesodermal marker genes *Xwnt-8* and *Xvent-1* was significantly reduced, suggesting that the dorsal fate, including head formation, was ectopically induced at the expense of ventral specification. *Xvent-2* expression was not reduced by HI-Xmsx-1 overexpression because Xvent-2 functions in a different BMP pathway from Xmsx-1. Also, marked suppression of a pan-mesodermal marker gene, *Xenopus brachyury* (*Xbra*), was observed, which may result in the truncation of the body axis, shown in Fig. II-3C. To my surprise, all of the anterior markers tested were induced by TI-Xmsx-1 as well in early gastrula, in spite of its inability to induce an ectopic head. I found, however, that in a late stage, *HoxB9* normally detected in posterior neural tissue was markedly downregulated in HI-Xmsx-1-injected embryos but not in TI-Xmsx-1-injected embryos (Fig. II-4). In addition, an another posterior marker *eFGF* which is significantly induced by TI-Xmsx-1 is not induced in HI-Xmsx-1-injected embryo. As an anterior marker *Xotx-2* was induced in both embryos at the late stage, it is suggested that differential regulation of the posterior marker gene expression may cause the different effects between HI- and TI-Xmsx-1.

To explain the ectopic head induction, I reasoned that a TGF-β-related ligand, *nodal*, might be ectopically induced in ventral blastomeres upon HI-Xmsx-1 overexpression, because *nodal* has been reported to be an inducer of head organizer genes (Piccolo et al., 1999; Zorn et al., 1999). Therefore, I next examined the expression level of nodal-related genes in the VMZ of HI-Xmsx-1-expressing embryos at early gastrula stage (St. 10.5).

However, RT-PCR analysis demonstrated no evident induction of nodal genes, *Xnr-1*, *Xnr-2*, *Xnr-3* and *Xnr-4* (data not shown). Consistent with this observation, in a reversed experiment the *Xnr* genes were not suppressed in the DMZ by Xmsx-1 overexpression (Fig. II-5).

Xmsx-1 antagonizes intracellular nodal signaling

Nodal expressed in early phase of blastula stage induces *cerberus* expression in the deep layer of dorsal marginal zone to form the anterior endomesoderm, implicating its role in head induction (Piccolo et al., 1999; Zorn et al., 1999). The ectopic head induction may indicate that HI-Xmsx-1 blocked the ability of ventrally localized endogenous Xmsx-1 activity to inhibit the nodal action at the intracellular signaling level. To investigate this possibility, I next tested whether Xmsx-1 could inhibit not the transcription but the intracellular signaling of nodal. Fig. II-6Ab shows a typical phenotype resulting from the ventral overexpression of nodal observed under this experimental conditions (Table II-1). It was previously reported that nodal alone could not induce a secondary axis with anterior structures such as brain and eyes, and that only by coinjecting nodal with noggin, could a complete secondary axis with a head be induced (Lustig et al., 1996). In contrast with the report, I found nodal to be sufficient to induce a complete secondary axis with organized head structures (Fig. II-6Ab) resembling the secondary axis induced by the ventral overexpression of Xwnt-8 or β-catenin mRNA (McMahon and Moon, 1989; Zeng et al., 1997). Increasing doses of Xmsx-1 gradually inhibited the formation of the head and secondary axis in a dose-dependent manner (Fig. II-6Ac, d, Table

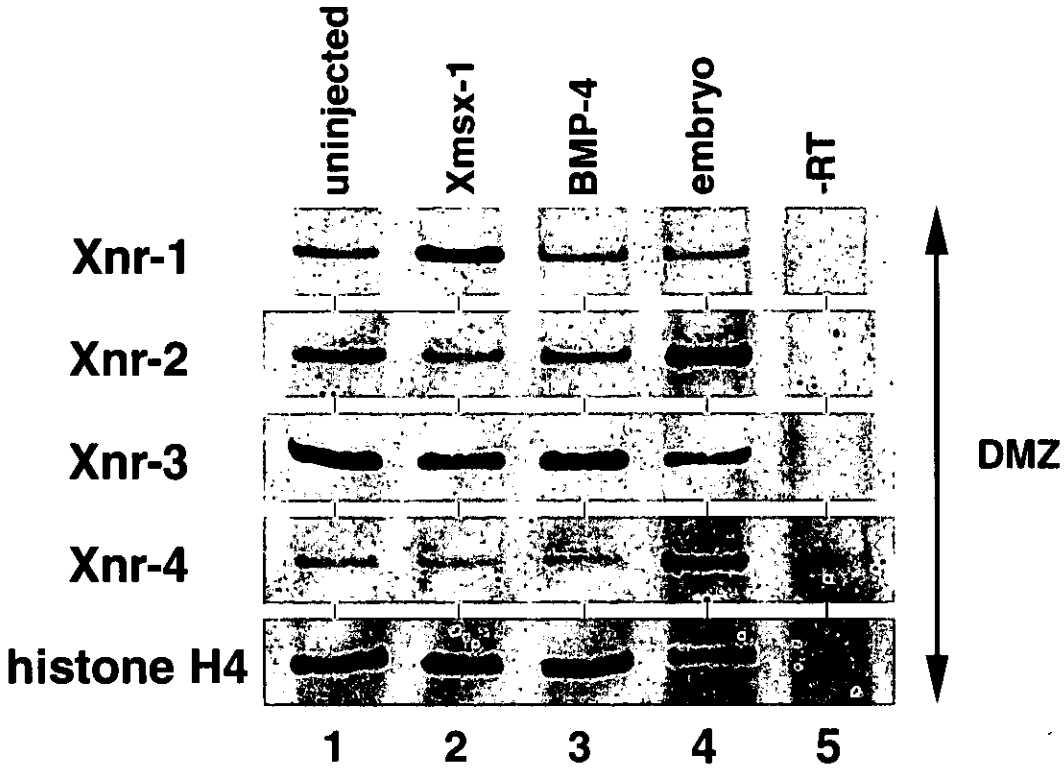


Fig. II-5. Xmsx-1 does not suppress nodal-related gene expression.
Embryos were either uninjected (lanes 1, 4, 5) or injected with 500 pg of Xmsx-1 (lane 2) or 200 pg of BMP-4 (lane 3) mRNA into the equatorial region of the two dorsal blastomeres at the 4-cell stage. DMZ (lanes 1, 2, 3) were cultured until sibling embryos reached stage 10.5, then nodal-related gene expression was analyzed by RT-PCR.

Table II-1.
Ectopic head formation by HI-Xmsx-1 and complete axis formation by Xnr-1

	mRNAs ventrally injected	Survivor	Phenotype %					
		% (n)	complete ectopic axis	ectopic head with eye and cement gland	ectopic head with cement gland	incomplete ectopic trunk axis	ectopic tail	normal
I	TI-Xmsx-1 (500 pg)	96.6 (28)	-	-	-	82.8	-	-
	HI-Xmsx-1 (500 pg)	96.8 (30)	-	29.0	54.8	-	-	-
II	HI-Xmsx-1 (100 pg)	96.7 (29)	-	30.0	3.3	63.3	-	-
	HI-Xmsx-1 (100 pg)+Xmsx-1 (1 ng)	90.6 (29)	-	-	-	-	9.4	62.5
III	Xnr-1 (100 pg)	100.0 (32)	68.8	-	-	15.6	-	3.1
	Xnr-1 (100 pg)+Xmsx-1 (250 pg)	100.0 (36)	-	2.8	-	91.7	-	-
	Xnr-1 (100 pg)+Xmsx-1 (500 pg)	96.9 (31)	-	-	-	6.3	-	71.9
IV	Xnr-1 (100 pg)	100.0 (32)	84.4	-	-	15.6	-	-
	Xnr-1 (100 pg)+BMP-4 (200 pg)	100.0 (35)	-	-	-	5.7	-	94.3

Phenotypes were estimated after 60 hours of development.
Other abnormalities including dorso-posterior defect, incomplete invagination and spontaneous ventralization are not described.

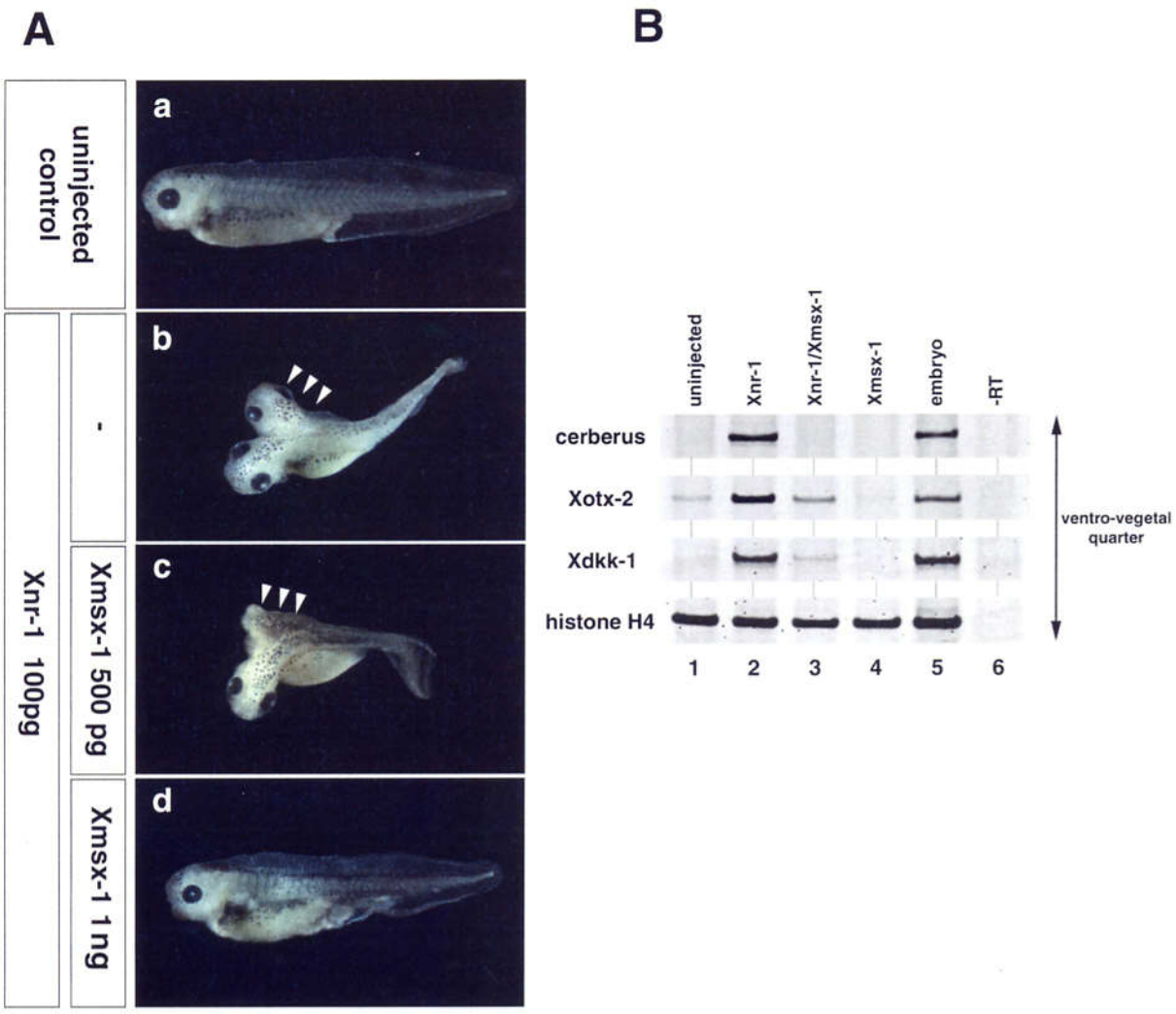


Fig. II-6. Xmsx-1 inhibits the Xnr-1 signaling for head formation.
(A) Typical phenotypes of Xnr-1 and Xmsx-1 mRNA-injected embryos. Xnr-1 mRNA (100 pg) was either injected (b) or coinjected with increasing amounts (c, 500 pg; d, 1 ng) of Xmsx-1 mRNA into the ventral marginal region of 4-cell stage embryos. Xmsx-1 suppressed the Xnr-1-induced complete secondary axis formation. (a) shows an uninjected control. (B) Gene expression analysis of head organizer markers in the ventral endomesoderm by RT-PCR. Xnr-1 and Xmsx-1 mRNAs were injected into the ventral marginal region of 4-cell-stage embryos and the ventro-vegetal quarters were dissected at stage 10.25 (lanes 1, 2, 3 and 4). Lane 1, uninjected control; lane 2, injected with 100 pg of Xnr-1 mRNA; lane 3, coinjected with 100 pg of Xnr-1 and 500 pg of Xmsx-1 mRNAs; lane 4, injected with 500 pg of Xmsx-1 mRNA. Lanes 5 and 6, uninjected whole embryo control, with (lane 5) or without (lane 6) the RT step.

II-1). Consistent with the observed reduction of the ectopic head and secondary axis were changes in marker gene expression; the head organizer genes *cerberus*, *Xotx-2*, and *Xdkk-1*, which were ectopically induced in the ventral endomesoderm by the nodal gene product Xnr-1, were efficiently down-regulated by Xmsx-1 (Fig. II-6B). This result suggests that Xmsx-1 does not change the expression level of *nodal* but inhibits its signaling. This interpretation might also explain the marked truncation of the primary axis (Fig. II-3C), because the nodal signal in early phases of development is known to be required to induce and/or maintain *Xbra* expression (Piccolo et al., 1999).

In addition, I next tested the effect of Xmsx-1 and HI-Xmsx-1 using a reporter gene for activin/nodal signaling. This reporter gene is derived from the activin responsive element (ARE) of the *goosecoid* promoter and is known to be activated by activin or nodal through a transcriptional complex that includes FAST, Smad2, and Smad4 (Chen et al., 1996a; Chen et al., 1997; Watabe et al., 1995). As shown in Fig. II-7A, the reporter-luciferase was preferentially activated in dorsal blastomeres, most likely reflecting dorsally localized nodal signaling in the embryo, while it was only weakly activated in the ventral side of the embryo. However, the luciferase activity in the ventral side was markedly enhanced in a dose-dependent manner by the injection of HI-Xmsx-1 mRNA. The dorsal activation of the reporter was significantly suppressed by the coinjection of wild-type Xmsx-1 (Fig. II-7B), suggesting that Xmsx-1 inhibits the transcription of nodal target genes. Interestingly, this suppression was restored by coinjecting Xnr-1 mRNA along with Xmsx-1. Taken together,

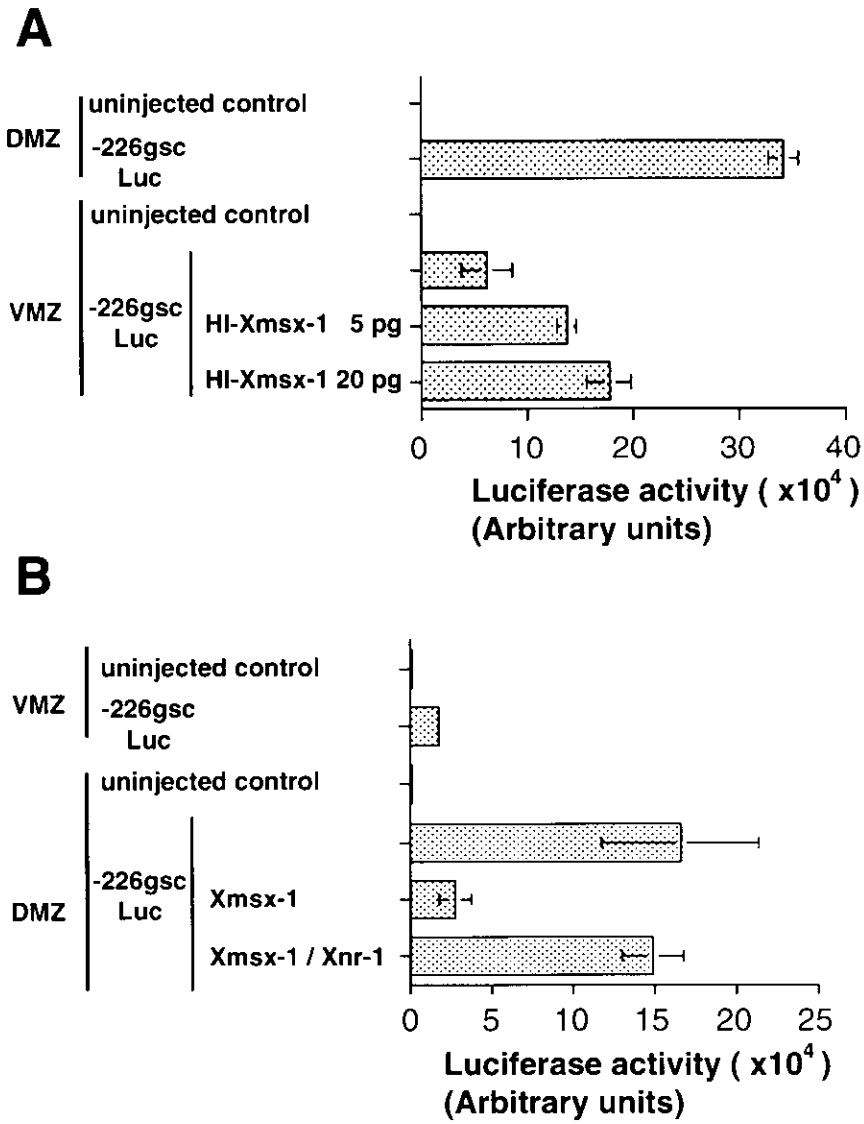


Fig. II-7. The activin responsive element (ARE) is activated by HI-Xmsx-1 and suppressed by Xmsx-1.
The construct -226gsc/Luc, which contains the luciferase gene under the control of the 226-bp *goosecoid* promoter containing the ARE was either injected alone or coinjected with the indicated mRNAs into the DMZ or VMZ of 4-cell-stage embryos. The injected or uninjected marginal zones were dissected at stage 10.25 (A) or 10 (B) and subjected to luciferase assays. (A) HI-Xmsx-1 activated the ARE in the ventral marginal region. (B) Xmsx-1 suppressed ARE activation in the dorsal side. Xnr-1 antagonized the suppressing activity of Xmsx-1 for the transcription through the ARE. Actual luciferase counts are presented.

these results strongly suggest that intracellular nodal signaling is inhibited by Xmsx-1 in vivo.

Xmsx-1 inhibits the transcriptional complex formation of Smad2/4 and FAST-1

How is the inhibitory mechanisms of Xmsx-1 for nodal signaling? It has been shown that, following the activation of the nodal receptors ActRIA/IB, and ActRII, and an EGF-CFC coreceptor (one eyed pinhead, and crypto/criptic for zebrafish and mouse, respectively), formation of a transcriptional complex is essential to activate nodal target genes. This complex includes Smad2, Smad4, and a winged helix transcription factor FAST, a protein whose binding site is the essential minimal enhancer sequence in the ARE (Watanabe and Whitman, 1999). This led me to speculate that Xmsx-1 may physically interact with the transcriptional complex triggered by nodal signaling. I tested whether Xmsx-1 protein binds the pathway-restricted Smads, Xsmad1, Xsmad2 (Baker and Harland, 1996; Graff et al., 1996), or the common Smads Xsmad4α and Xsmad4β (Howell et al., 1999; Masuyama et al., 1999). I expressed Flag-tagged Xmsx-1 protein and HA-tagged Smad proteins transiently in 293T cells, and the cell extracts were subjected to immunoprecipitation with anti-Flag antibodies, followed by Western blot analysis with anti-HA antibodies. Interestingly, all of the Smads were coprecipitated with Xmsx-1, although the efficacy varied (Fig. II-8A). Among them, Xsmad4β was most efficiently immunoprecipitated with Xmsx-1. This may be due to the fact that

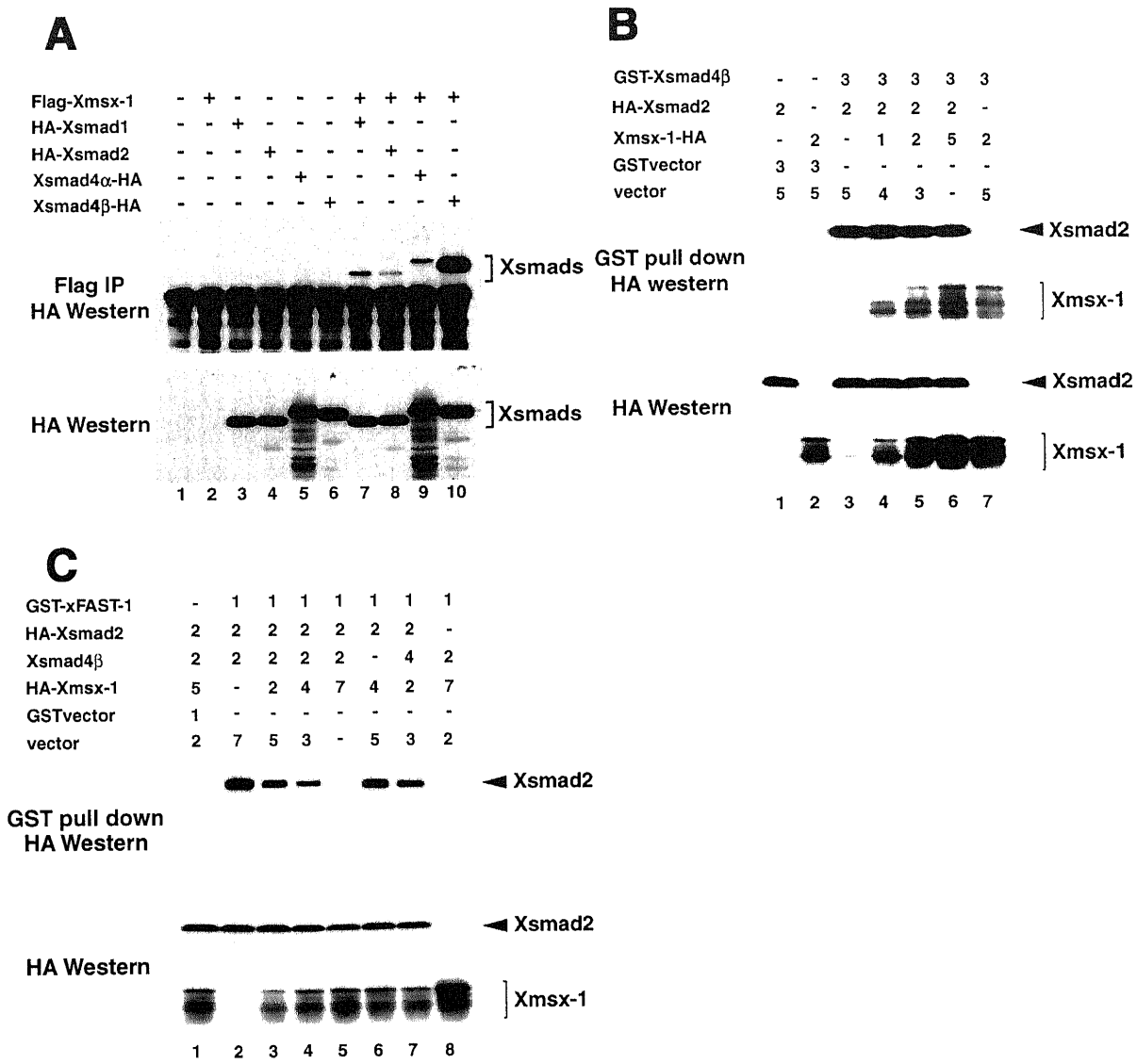


Fig. II-8. Xmsx-1 physically interacts with Smads.
(A) Xmsx-1/Xsmads complexes were detected by immunoprecipitation. 293T cells were transiently transfected with Flag-tagged Xmsx-1 alone (lane 2) or the indicated HA-tagged Xsmads alone (lanes 3-6), or cotransfected with Flag-tagged Xmsx-1 and each HA-tagged Xsmad (lanes 7-10). Lysates were assayed either by immunoprecipitation with an anti-Flag antibody followed by Western blotting with an anti-HA antibody (upper panel) or by Western blotting the whole lysate with an anti-HA antibody (lower panel). Lane 1 is a nontransfected control. (B) Xmsx-1 additively participates in the Xsmad2/Xsmad4β complex. 293T cells were transiently cotransfected with GST-tagged Xsmad4β and HA-tagged Xsmad2 together with increasing amounts of HA-tagged Xmsx-1 (lanes 3-6). Lysates were assayed either by GST pull-down analysis followed by Western blotting with an anti-HA antibody (upper panel) or by Western blotting the whole lysate with an anti-HA antibody (lower panel). As negative controls, GSTvector expressing GST protein alone was cotransfected either with HA-tagged Xsmad2 (lane 1) or with HA-tagged Xmsx-1 (lane 2). (C) Xmsx-1 excludes xFAST-1 from Xsmad2/4 complex in a dose-dependent manner. Cells were cotransfected with the indicated amount of GST-xFAST-1, HA-Xsmad2, Xsmad4β and HA-Xmsx-1 constructs. Lysates were assayed as above in (B).

Smad4β is localized exclusively to nucleus at a higher concentration, while the other Smads are present in both the cytoplasm and the nucleus. To investigate the possibility that the binding of Xmsx-1 to Smad4 replaces the activin/nodal-regulated Smad2, I examined whether increasing doses of Xmsx-1 could change Smad2 level in the complex (Fig. II-8B). However, the level of Smad2 in the complex did not change as a result of Xmsx-1 expression. This indicates that Xmsx-1 is likely to bind to Smad2/4 complex additively. Furthermore, in this context I tested whether the incorporation of Xmsx-1 into Smad2/4 complex could exclude the transcription factor FAST. GST-tagged xFAST-1, HA-tagged Xsmad2 and increasing dose of HA-tagged Xmsx-1 were expressed in 293T cell in the presence or absence of Xsmad4β and subjected to GST pull down analysis (Fig. II-8C). The results showed that Xmsx-1 did not bind to xFAST-1 (Fig. II-8C, lane 8) but clearly inhibited xFAST-1 to bind Xsmad2 in a dose-dependent manner (Fig. II-8C, lanes 2-5). Interestingly, this competitive inhibition depended on the presence of Xsmad4β (Fig. II-8C, lanes 4 and 6). These results suggest that the binding of Xmsx-1 to Smad4 may exclude FAST from the Smad2/4 complex and this mechanism may explain how the transcriptional activity of the complex is negatively regulated.

Endogenous BMP signaling in the endoderm

If BMP/Xmsx-1 signaling is indeed necessary to repress head formation in vivo through the inhibition of head organizer genes, the activities of BMP and Xmsx-1 should be present in the ventral endoderm and absent in the

anterior endoderm where the head organizer genes are expressed. To address this question, I localized BMP signaling using a anti-phosphoSmad1 antibody that preferentially recognizes the phosphorylated (active) form of BMP-regulated Smads, Smad1 and Smad5 (Faure et al., 2000; Kurata et al., 2000; Persson et al., 1998).

As predicted, BMP signaling represented by the anti-phosphoSmad1 staining was detected in the ventral endoderm region but not in the anterior endoderm (Fig. II-9A). Consistent with this, Xmsx-1 was also detected immunohistochemically in the overlapping region with its specific antibodies (Fig. II-9B). These results further support the idea that BMP/Xmsx-1 activities are necessary in the ventral endoderm to repress head formation and that both activities are absent in the corresponding dorsal region, where the head organizer is formed.

Extensive inhibition of BMP signaling is sufficient for head formation

I showed that a dominant-inhibitory form of a homeobox gene Xmsx-1 that faithfully reflects BMP activity alone is sufficient to induce ectopic head. These results indicate that inhibition of BMP signaling alone should also result in head formation. This raised an interesting question as to why inhibition of BMP by tBR in the ventral side never induces a complete head but only a partial dorsal axis (Suzuki et al., 1994). As there are several BMP subfamily members such as BMP-2, BMP-4 and BMP-7, there are multiple cell surface receptors transducing their signals in *Xenopus* embryo. At least three type I receptors, BMPRIA (ALK3), BMPRIB (ALK6), and ActRIA (ALK2) and three type II receptors, ActRII, ActRIIB, and

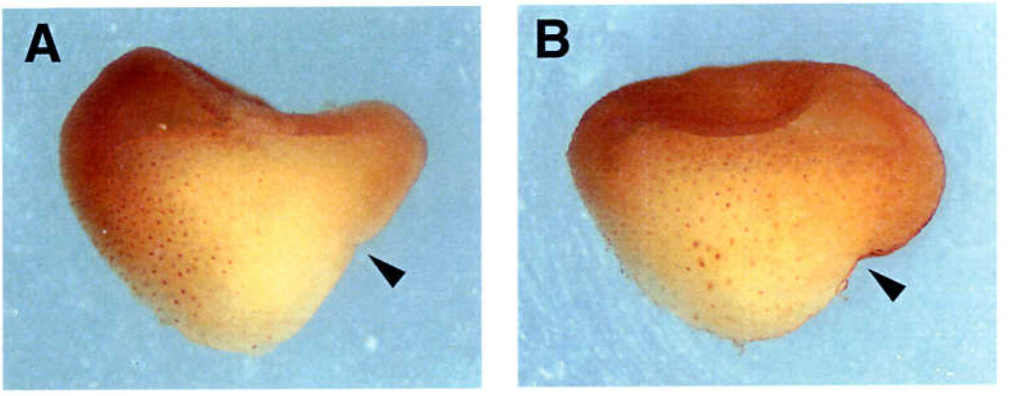


Fig. II-9. Immunostaining of activated Smad1 and Xmsx-1 protein.
Gastrula embryos were subjected to immunohistochemistry using anti-phosphoSmad1 (A) and anti-MSX-1 (B) antibodies. Embryos were bisected along the dorsoventral axis. The dorsal blastopore lip is indicated with an arrow.

BMPRII have so far been identified as receptors mediating the BMP signal (Dale and Jones, 1999). Due to the wide range of BMP activities generated by the subtypes, I reasoned that the previous experiments inhibiting BMP signals with tBR were not complete. In this context, I attempted to block BMP activities as extensively as possible using several combinations of dominant-negative receptors, or BMP antagonists. I first coinjected the mRNAs for tBR and truncated BMPRII. As shown in Fig. II-10A, no head structure was formed in the ectopic dorsal axis of the injected embryos. Next I coinjected a truncated ALK2 mRNA in addition to the former combination of mRNA because ALK2 is known to contribute to BMP-7 signaling. As shown in Fig. II-10B, an almost complete head with eye and a cement gland was induced in the injected embryos (Table II-2). The head induction rate appears to be depending on the inhibitory spectrum of BMP signals. This suggests that BMP has only to be blocked extensively for head induction. To confirm this notion, I also examined the combined effect of so-called organizer factors noggin, chordin and follistatin, each of which is insufficient to induce ectopic head by ventral overexpression (Fig. II-10C). As I expected, double or triple mRNA injection led to an ectopic head with eyes and a cement gland (Fig. II-10D, Table II-2), supporting the prior result with the dominant-negative receptors. Taking account of the results using HI-Xmsx-1, I propose here that inhibition of BMP/Xmsx-1 activities is not only necessary but sufficient to induce head formation.

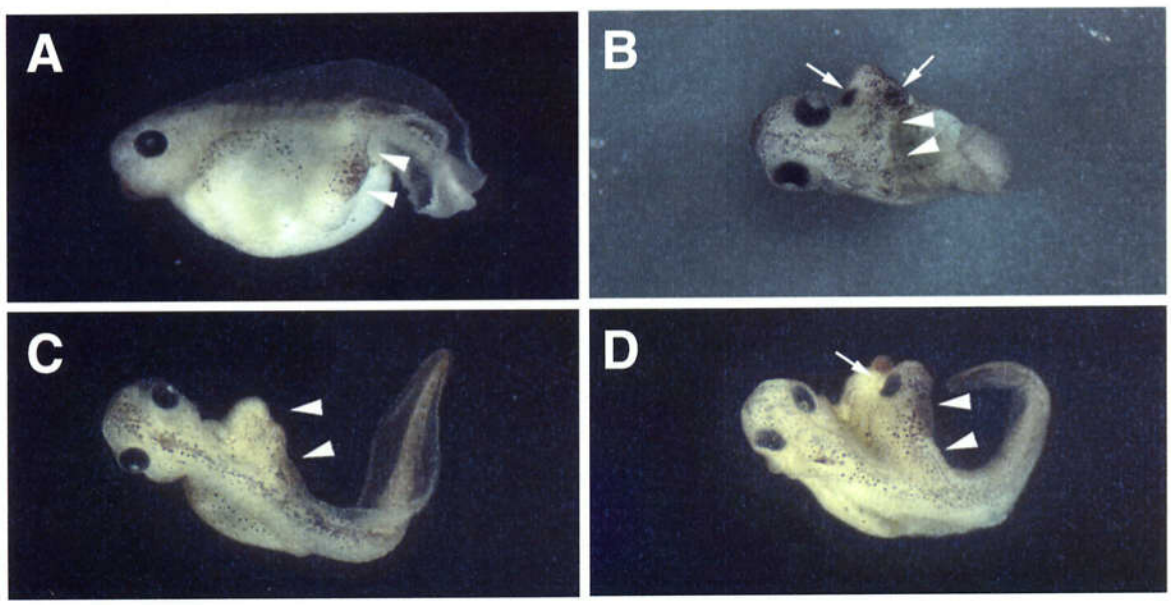


Fig. II-10. Phenotype of embryos ventrally overexpressing inhibitors of BMP signaling.
Four-cell stage *Xenopus* embryos were ventrally microinjected with the following synthetic mRNAs; (A) 200 pg of tBR and 500 pg of truncated BMPRII mRNAs or (B) together with 100 pg of truncated ALK2 mRNA; (C) 300 pg of chordin mRNA alone; (D) 100 pg of chordin, 0.3 pg of noggin and 20 pg of follistatin mRNAs. Both triple mRNA injections efficiently led to an ectopic head with eyes (white arrow) and a cement gland while injection of increasing amount of any single mRNA did not induce an ectopic head structure. White arrowheads, secondary trunk.

Table II-2.
Extensive inhibition of BMP activity induces ectopic head structure

	mRNAs ventrally injected	Survivor	Phenotype %					
		% (n)	complete ectopic axis	ectopic head with eye and cement gland	ectopic head with cement gland	incomplete ectopic trunk axis	ectopic tail	normal
I	tBRIA (200 pg)+tBRII (500 pg)	93.5 (29)	-	-	-	93.5	-	-
	tBRII (500 pg)+tALK2 (100 pg)	96.7 (29)	-	-	-	83.3	6.7	-
	tALK2 (100 pg)+tBRIA (200 pg)	100.0 (30)	-	-	-	93.3	-	3.3
	tBRIA (200 pg)+tBRII (500 pg)+tALK2 (100 pg)	97.0 (32)	-	42.4	6.1	45.5	-	-
II	chordin (300 pg)	100.0 (34)	-	-	-	67.6	29.4	2.9
	noggin (1 pg)	100.0 (35)	-	-	-	94.3	2.9	-
	follistatin (60 pg)	96.9 (31)	-	-	-	81.3	-	-
	chordin (150 pg)+noggin (0.5 pg)	96.9 (31)	18.8	-	-	75.0	-	-
	noggin (0.5 pg)+follistatin (30 pg)	96.8 (30)	45.2	-	-	32.3	-	3.2
	follistatin (30 pg)+chordin (150 pg)	93.3 (28)	-	-	-	83.3	3.3	-
	chordin (100 pg)+noggin (0.3 pg)+follistatin (20 pg)	93.5 (29)	74.2	-	-	19.4	-	-

Phenotypes were estimated after 60 hours of development.
Other abnormalities including dorso-posterior defect, incomplete invagination and spontaneous ventralization are not described.
tBRIA, truncated BMPRIA; tBRII, truncated BMPRII; tALK2, truncated ALK2.

Discussion

Is the inhibition of BMP activity sufficient for head induction?

One of the most important observations of this report was that HI-Xmsx-1 induced an ectopic head. The general belief has been that inhibition of BMP signaling, for example by a truncated BMP type I receptor (BMPRIA/ALK3), is not sufficient to induce a complete secondary body axis with a head structure (Suzuki et al., 1994). In addition, recent studies have suggested that inhibition of all three polypeptide growth factors, BMP, nodal, and Wnts, is necessary for head formation (Glinka et al., 1997; Piccolo et al., 1999). Dkk-1, which was identified as a Wnt antagonist, is capable of inducing an ectopic head when combined with the action of the truncated BMP receptor, tBR (Glinka et al., 1998). Cerberus, originally found to be a head inducer, was later found to bind all of the above three factors to inhibit their function (Piccolo et al., 1999). In contrast, in this study, I have found that the expression of HI-Xmsx-1, an inhibitor of Xmsx-1, alone is sufficient to induce an ectopic head. Because Xmsx-1 is believed to faithfully mimic the effects of BMP in *Xenopus* embryos (Suzuki et al., 1997b), this observation was unexpected and somewhat puzzling, considering the results of previous studies. One possible explanation is that inhibition of the BMP/Xmsx-1 pathway by HI-Xmsx-1 might ectopically activate nodal signaling in the anterior endomesoderm, which in turn could activate head organizer genes such as *cerberus*, *Xhex*, and *Xdkk-1* to lead to head formation. Alternatively or additionally, HI-Xmsx-1 might have acted during later developmental stages to inhibit the BMP signal required for the maintenance of *Xwnt-8* expression in the

VMZ; thus, both the BMP and Xwnt-8 signals would be inhibited, conferring a sufficient condition for ectopic head formation.

Previously, the ectopic overexpression of nodal was reported to induce only a partial secondary dorsal axis without a head, and it was shown to induce a head only when cooverexpressed with noggin (Lustig et al., 1996). However, I have clearly demonstrated here that nodal alone can induce a complete secondary axis with a head when it is ventrally overexpressed. Therefore, the former explanation is consistent with my functional analysis of nodal in the early *Xenopus* embryo. It is also known that, later in development, nodal must be inhibited in the dorsal side. This has been proposed to occur indirectly through the induction of *cerberus* (Piccolo et al., 1999). In addition, head induction by chordin and frz-b was also rescued by the coinjection of Xnr-1 DNA. Therefore, cerberus expressed ectopically by DNA injection may serve as an inhibitor of nodal in later stages fulfilling the requirement for head formation. When HI-Xmsx-1 was dorsally injected, defect in head formation was observed (data not shown). This may be due to the hyperactivation of nodal signaling and is consistent with the above speculation.

The latter possibility, involving the down-regulation of *Xwnt-8*, is also likely. A ventral mesoderm marker, *Xwnt-8* was significantly down-regulated as a result of the inhibition of Xmsx-1 activity. It is believed that the BMP activity, most likely through its induction of Xmsx-1 activity (Takeda et al., 2000), is necessary for the onset and maintenance of the ventral expression of *Xwnt-8* at the early gastrula stage. The results here further confirmed that termination of the BMP signaling cascade with HI-

Xmsx-1 leads to the loss of *Xwnt-8* expression. Despite the similar inhibition of both BMP and Xwnt-8 activity, the mechanism of head induction by Dkk-1 and tBR (Glinka et al., 1998) may be slightly different because the inhibition of Wnts and BMP by Dkk-1 and tBR is thought to be solely an extracellular event, while HI-Xmsx-1 inhibits BMP signals intracellularly and *Xwnt-8* at the transcriptional level.

To investigate the above possibilities, I expressed HI-Xmsx-1 using a DNA vector, with the intention that HI-Xmsx-1 be expressed later than it would be by mRNA injection. Interestingly, ectopic induction of the head was not observed (data not shown), indicating that early action of HI-Xmsx-1 is essential for ectopic head induction, which would support the former possibility. Also it was previously demonstrated that the blockage of BMP and anti-dorsalizing morphogenetic protein (ADMP), a member of TGF-β superfamily expressed in the *Xenopus* trunk organizer, induces head formation (Dosch and Niehrs, 2000). To address whether the ectopic head induction by HI-Xmsx-1 was due to the inhibition of ADMP, I tested the effect of HI-Xmsx-1 on the ventralizing phenotype by ADMP. No antagonizing ability of HI-Xmsx-1 was observed against ADMP (data not shown). Furthermore, ADMP expression level was not repressed in DMZ, while it is induced in VMZ by HI-Xmsx-1 (data not shown). These suggests that HI-Xmsx-1 induced an ectopic head not through the ADMP inhibition.

As shown in Fig. II-10, I demonstrated that inhibition of extensive spectrum of BMP activities using multiple truncated BMP receptors or three extracellular BMP antagonists, so-called organizer factors, could form

an ectopic head structure, often with eyes and a cement gland, suggesting that the incomplete secondary axis formation observed in previous studies was due to residual endogenous BMP signals. Therefore it appears that BMP/Xmsx-1 signaling play a role not only as trunk repressor but also as head repressor in vivo.

Xmsx-1 inhibits nodal signaling.

I showed that Xmsx-1 inhibited the activation of an ARE reporter gene in the dorsal blastomeres of *Xenopus* embryos. I therefore conclude that wild-type Xmsx-1 inhibits intracellular nodal signaling. This result may in turn suggest that HI-Xmsx-1 might have activated ectopic nodal signaling when overexpressed in ventral blastomeres. In other words, potential nodal signaling may be present in the ventral side of the embryo, as has been shown that nodal transcript is evenly distributed in early embyo (Jones et al., 1995), but is inactivated by the BMP/Xmsx-1 signal. In fact, endogenous activated Smad2 is detected in the ventral endoderm as well as in the anterior endoderm of gastrula stage embryo (Faure et al., 2000). In this study, I hypothesized that this inhibition occurs at the intracellular signaling level. I also revealed that an increasing level of Xmsx-1 could efficiently exclude FAST protein from Smad2/4 complex depending on the presence of Smad4. Taken together, it is speculated that through the binding to Smad4, Xmsx-1 associates with Smad2 and inhibits FAST-induced transcription. However, the exact mechanism in vivo by which nodal signaling is preferentially inhibited by the binding of Xmsx-1 to the complex remains to be investigated.

In this study, I clearly showed that Xmsx-1 inhibit nodal at the intracellular signaling level in the ventral endoderm without apparent change in the level of nodal transcripts. Recently, however, the expression of *nodal* was shown to be autoinduced by nodal signaling through a FAST-regulated module in the first intron of the gene (Osada et al., 2000). This indicates that the modulation of nodal signaling should lead to change in expression level of the ligand, which was not the case in this study. One possibility to explain this discrepancy is the fact that antivin/lefty, which is a member of TGF-β superfamily and the antagonist of nodal signaling acting in a negative feedback loop to suppress the maintenance of nodal ligand, is also induced by nodal signaling. By this mechanism, the expression level of nodal may be maintained at a constant level even when Xmsx-1 is overexpressed.

Finally, an important question is how universal among species the head repression mechanism proposed here is. Targeted disruption of mouse *Msx1* and/or *2* reveals no antero-posterior patterning defect, although developmental defects were found in several organs of each *Msx* gene disrupted mice (Satokata et al., 2000; Satokata and Maas, 1994). In the mouse, three *Msx* genes were isolated, *Msx1, 2* and *3*. Thus, *Msx3* or another related homeobox genes may act redundantly in the mutants, as functional redundancy was previously reported for Msx1 and 2 (Satokata et al., 2000). Alternatively, the head repression mechanism by BMP/Msx-1 may be specific to amphibian. I have shown that nuclear localization of Xmsx-1 as well as phosphorylated BMP-driven Smads in ventral endoderm but not in anterior endoderm (Fig. II-9A, B), supporting the

speculated mechanism of head induction (de Souza and Niehrs, 2000). However, expression of mouse Msx proteins in the primitive streak and absence in anterior visceral endoderm (AVE), has not been demonstrated.

CONCLUSIONS AND PERSPECTIVES TO THE FUTURE

The pattern formation along three dimensional axis in embryogenesis is the first step of the body plan after fertilization in vertebrate development. BMPs and nodal signals play crucial roles in the pattern formation of three germ layers, ectoderm, mesoderm, and endoderm.

In this thesis, I investigated the role of Xmsx-1 homeobox gene in the events as a downstream target of BMP signal in *Xenopus*. In Part I, I demonstrated that Xmsx-1 acts as a transcriptional repressor in the embryogenesis of *Xenopus* and is required for BMP-triggered ventralizing pathway in mesoderm and ectoderm. It is suggested that Xvent-1 acts downstream of Xmsx-1 and that Xvent-2 may be acting in ventralizing pathways independent of Xmsx-1, although both are induced by BMP and possess ventralizing activity. In Part II, I revealed that dominant interference of Xmsx-1 or extensive inhibition of BMP signaling is sufficient for head formation suggesting that BMP/Xmsx-1 signal is required to suppress head formation in the ventral side of embryo. I also suggested that Xmsx-1 acts as a posterializing factor through the inhibition of FAST-1 mediated nodal signaling by interacting with Smads. Recently, two novel nodal-related ligands, Xnr-5 and Xnr-6, were found to be expressed at the mid-blastula transition in dorsal-vegetal region including Nieuwkoop center, which has been believed in possessing the organizer inducing activity (Takahashi et al., 2000). In fact, Xnr-5 and Xnr-6 can induce other nodal-related ligands expressed in the organizer region. Taken together these recent studies, one possible mechanism of organizer

formation is; in normal development Xnr-5 and Xnr-6 expressed at mid-blastula transition induce *Xhex* in the endodermal region, and *Xnr-1* and *Xnr-2* in the endomesodermal region of dorsal side. In the end of blastula stage *cerberus* is induced in the Xhex expressing region and inhibits BMP and Xwnt-8 signals by binding their ligands directly. Xnr-1 and Xnr-2 may contribute to form organizer inducing BMP antagonists, noggin, chordin and follistatin, Wnt antagonist Xdkk in the surrounding region (Fig. 2A,B,C). I propose that Xmsx-1 may be involved in inhibition of all of these nodal signaling in the ventral side of three germ layer not to form both head organizer and trunk organizer by preventing the formation of FAST/Smads transcriptional activator complex (Fig. 2D,E). And I also propose that in head induction, extensive inhibition of BMP/Xmsx-1 signal in the early stage of organizer formation is impotant and consequently induces *Xdkk-1* and *cerberus* expression to inhibit Wnt signal.

Thus, the findings in this thesis suggest that Xmsx-1 plays a major role in BMP stimulated ventralization and head suppression. On the other hand Xvent-2 is also ventralizing factor and immediate early responsive gene product to BMP. Although I suggested that Xmsx-1 and Xvent-2 are acting in independent pathways, the differences of the role between the two pathways are remain to be investigated. At least one functional difference is that Xvent-2 can induce BMP-4 expression (Onichtchouk et al., 1996) while Xmsx-1 not (Part I). Further investigation is needed to elucidate the BMP-triggered ventralizing pathways. Several homeobox genes, including *Xmsx-1*, *Xmsx-2*, *Xvent-1*, *Xvent-2*, and *Xvex-1*, have been

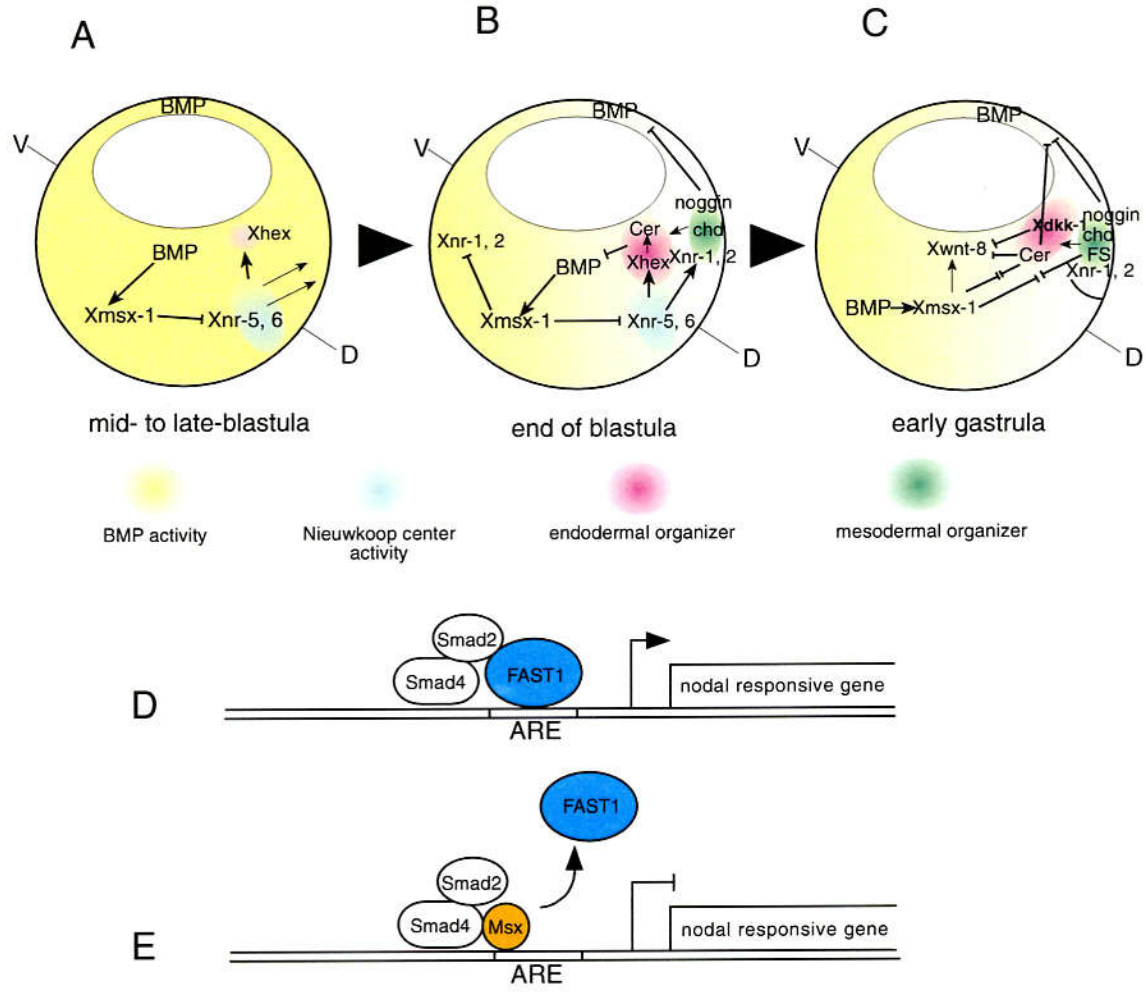


Fig. 3. Summary

(A, B, C) Model of molecular mechanisms in the formation and the inhibition of organizer activity. (A) In the mid blastula stage, expression of BMPs is detected ubiquitously in the embryo. At this stage Xnr-5 and Xnr-6 begin to express in the dorsal-vegetal region including the Nieuwkoop center and may contribute to form organizer activity in the endoderm and mesoderm regions of dorsal submarginal zone by activating Xhex gene and other nodal-related genes (Xnr-1 and Xnr-2). (B) At the end of blastula stage, cerberus is expressed in the endodermal organizer region to inhibit BMP and Wnt. In the mesodermal organizer region, noggin and chordin are induced by Xnr-1 and/or Xnr-2 to block BMP activity. (C) In early gastrula, follistatin and Xdkk-1 are expressed in the mesodermal and endomesodermal organizer region, respectively to inhibit BMP and Wnt signal. Xmsx-1 inhibits these nodal signalings in the ventral side of three germ layers. (D, E) Xmsx-1 is thought to inhibit the nodal signal by binding to Smads to exclude FAST-1 from the transcriptional activator complex, FAST-1/Smad2/Smad4, depending on the ARE.

shown to express in early Xenopus development responding to BMP and act as ventralizing factors. One of the most interesting features is that all of these factors have been suggested to function as transcriptional repressors. It is tempting to speculate that ventralization is the result of suppression of dorsalizing factor genes expression activated by the dorsal morphogen including nodal related factors. Future studies are to be aimed at the molecular mechanisms how these ventralizing transcriptional repressors trigger downstream events leading to ventral differentiation of embryonic tissues.

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REFFERENCES

Artinger, M., Blitz, I., Inoue, K., Tran, U. and Cho, K. W. (1997). Interaction of goosecoid and brachyury in Xenopus mesoderm patterning. *Mech Dev* **65**, 187-96.

Asashima, M., Nakano, H., Shimada, K., Kinoshita, K., Ishii, K., Shibai, H. and Ueno, N. (1990). Mesoderm induction in early amphibian embryos by activin A (erythroid differentiation factor). *Roux's Arch. Dev. Biol.* **198**, 330-335.

Ault, K. T., Dirksen, M. L. and Jamrich, M. (1996). A novel homeobox gene PV.1 mediates induction of ventral mesoderm in Xenopus embryos. *Proc. Natl. Acad. Sci. U S A* **93**, 6415-20.

Baker, J. C. and Harland, R. M. (1996). A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. *Genes Dev* **10**, 1880-9.

Beddington, R. S. and Robertson, E. J. (1999). Axis development and early asymmetry in mammals. *Cell* **96**, 195-209.

Boncinelli, E. (1997). Homeobox genes and disease. *Curr. Opin. Genet. Dev.* **7**, 331-7.

Boonstra, J., Rijken, P., Humbel, B., Cremers, F., Verkleij, A. and van Bergen en Henegouwen, P. (1995). The epidermal growth factor. *Cell Biol Int* **19**, 413-30.

Botas, J. (1993). Control of morphogenesis and differentiation by HOM/Hox genes. *Curr. Opin. Cell Biol.* **5**, 1015-22.

Bouwmeester, T. (2001). The Spemann-Mangold organizer: the control of fate specification and morphogenetic rearrangements during gastrulation in Xenopus. *Int J Dev Biol* **45**, 251-8.

Chen, X., Rubock, M. J. and Whitman, M. (1996a). A transcriptional partner for MAD proteins in TGF-beta signalling [published erratum appears in Nature 1996 Dec 19-26;384(6610):648]. *Nature* **383**, 691-6.

Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G. and Whitman, M. (1997). Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature* **389**, 85-9.

Chen, Y., Bei, M., Woo, I., Satokata, I. and Maas, R. (1996b). Msx1 controls inductive signaling in mammalian tooth morphogenesis. *Development* **122**, 3035-44.

Cho, K. W., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid. *Cell* **67**, 1111-20.

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991). Xwnt-8, a Xenopus Wnt-1/int-1-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111**, 1045-55.

Christian, J. L. and Moon, R. T. (1993). Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of Xenopus. *Genes Dev.* **7**, 13-28.

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B. and Robertson, E. J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* **120**, 1919-28.

Conlon, F. L., Sedgwick, S. G., Weston, K. M. and Smith, J. C. (1996). Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. *Development* **122**, 2427-35.

Dale, L., Howes, G., Price, B. M. and Smith, J. C. (1992). Bone morphogenetic protein 4: a ventralizing factor in early Xenopus development. *Development* **115**, 573-85.

Dale, L. and Jones, C. M. (1999). BMP signalling in early Xenopus development. *Bioessays* **21**, 751-60.

Davidson, D. (1995). The function and evolution of Msx genes: pointers and paradoxes. *Trends Genet.* **11**, 405-11.

de Souza, F. S. and Niehrs, C. (2000). Anterior endoderm and head induction in early vertebrate embryos. *Cell Tissue Res* **300**, 207-17.

Derynck, R., Zhang, Y. and Feng, X. H. (1998). Smads: transcriptional activators of TGF-beta responses. *Cell* **95**, 737-40.

Dosch, R. and Niehrs, C. (2000). Requirement for anti-dorsalizing morphogenetic protein in organizer patterning. *Mech Dev* **90**, 195-203.

Fagotto, F., Guger, K. and Gumbiner, B. M. (1997). Induction of the primary dorsalizing center in Xenopus by the Wnt/GSK/beta-catenin signaling pathway, but not by Vg1, Activin or Noggin. *Development* **124**, 453-60.

Fainsod, A., Steinbeisser, H. and De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. *Embo J.* **13**, 5015-25.

Faure, S., Lee, M. A., Keller, T., ten Dijke, P. and Whitman, M. (2000). Endogenous patterns of TGFbeta superfamily signaling during early Xenopus development. *Development* **127**, 2917-31.

Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schier, A. F. and Talbot, W. S. (1998). Zebrafish organizer development and germ-layer formation require nodal- related signals [see comments]. *Nature* **395**, 181-5.

Ferreiro, B., Artinger, M., Cho, K. and Niehrs, C. (1998). Antimorphic goosecoids. *Development* **125**, 1347-59.

Foerst-Potts, L. and Sadler, T. W. (1997). Disruption of Msx-1 and Msx-2 reveals roles for these genes in craniofacial, eye, and axial development. *Dev. Dyn.* **209**, 70-84.

Friedman, A. D., Triezenberg, S. J. and McKnight, S. L. (1988). Expression of a truncated viral trans-activator selectively impedes lytic infection by its cognate virus. *Nature* **335**, 452-4.

Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C. (1995). Antagonizing the Spemann organizer: role of the homeobox gene Xvent-1. *Embo J.* **14**, 6268-79.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-62.

Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C. and Niehrs, C. (1997). Head induction by simultaneous repression of Bmp and Wnt signalling in Xenopus. *Nature* **389**, 517-9.

Gotoh, Y. and Nishida, E. (1995). Activation mechanism and function of the MAP kinase cascade. *Mol Reprod Dev* **42**, 486-92.

Graff, J. M., Bansal, A. and Melton, D. A. (1996). Xenopus Mad proteins transduce distinct subsets of signals for the TGF beta superfamily. *Cell* **85**, 479-87.

Graham, A., Francis-West, P., Brickell, P. and Lumsden, A. (1994). The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. *Nature* **372**, 684-6.

Han, K. and Manley, J. L. (1993). Transcriptional repression by the Drosophila even-skipped protein: definition of a minimal repression domain. *Genes Dev.* **7**, 491-503.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol* **36**, 685-95.

Heasman, J. (1997). Patterning the Xenopus blastula. *Development* **124**, 4179-91.

Hemmati-Brivanlou, A., Frank, D., Bolce, M. E., Brown, B. D., Sive, H. L. and Harland, R. M. (1990). Localization of specific mRNAs in Xenopus embryos by whole-mount in situ hybridization. *Development* **110**, 325-30.

Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-95.

Hemmati-Brivanlou, A. and Thomsen, G. H. (1995). Ventral mesodermal patterning in Xenopus embryos: expression patterns and activities of BMP-2 and BMP-4. *Dev. Genet.* **17**, 78-89.

Hogan, B. L. M. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *GENES & DEVELOPMENT* **10**, 1580-1594.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1991). Xenopus Myf-5 marks early muscle cells and can activate muscle genes ectopically in early embryos. *Development* **111**, 551-60.

Howell, M., Itoh, F., Pierreux, C. E., Valgeirsdottir, S., Itoh, S., ten Dijke, P. and Hill, C. S. (1999). Xenopus Smad4beta is the co-Smad component of developmentally regulated transcription factor complexes responsible for induction of early mesodermal genes. *Dev Biol* **214**, 354-69.

Iemura, S., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H. and Ueno, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc. Natl. Acad. Sci. U S A* **95**, 9337-42.

Itoh, K. and Sokol, S. Y. (1999). Axis determination by inhibition of Wnt signaling in Xenopus. *Genes Dev* **13**, 2328-36.

Jans, D. A. and Hassan, G. (1998). Nuclear targeting by growth factors, cytokines, and their receptors: a role in signaling? *Bioessays* **20**, 400-11.

Jones, C. M., Kuehn, M. R., Hogan, B. L., Smith, J. C. and Wright, C. V. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* **121**, 3651-62.

Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. and Hogan, B. L. (1992). DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in Xenopus mesoderm induction. *Development* **115**, 639-47.

Kao, K. R. and Elinson, R. P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced Xenopus laevis embryos. *Dev. Biol.* **127**, 64-77.

Kelley, C., Blumberg, H., Zon, L. I. and Evans, T. (1993). GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. *Development* **118**, 817-27.

Kessler, D. S. and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* **121**, 2155-64.

Klymkowsky, M. W. and Hanken, J. (1991). Whole-mount staining of Xenopus and other vertebrates. *Methods Cell Biol* **36**, 419-41.

Kurata, T., Nakabayashi, J., Yamamoto, T. S., Mochii, M. and Ueno, N. (2000). Visualization of endogenous BMP signaling during *Xenopus* development. *Differentiation* **67**, 33-40.

Ladher, R., Mohun, T. J., Smith, J. C. and Snape, A. M. (1996). Xom: a Xenopus homeobox gene that mediates the early effects of BMP-4. *Development* **122**, 2385-94.

Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D. and Moon, R. T. (1997). Establishment of the dorso-ventral axis in Xenopus embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J Cell Biol* **136**, 1123-36.

Laurent, M. N. and Cho, K. W. (1999). Bone morphogenetic protein antagonism of Spemann's organizer is independent of Wnt signaling. *Dev. Biol.* **206**, 157-62.

Lustig, K. D., Kroll, K., Sun, E., Ramos, R., Elmendorf, H. and Kirschner, M. W. (1996). A Xenopus nodal-related gene that acts in synergy with noggin to induce complete secondary axis and notochord formation. *Development* **122**, 3275-82.

Maas, R., Chen, Y. P., Bei, M., Woo, I. and Satokata, I. (1996). The role of Msx genes in mammalian development. *Ann. N Y Acad. Sci.* **785**, 171-81.

Maeda, R., Kobayashi, A., Sekine, R., Lin, J. J., Kung, H. and Maeno, M. (1997). Xmsx-1 modifies mesodermal tissue pattern along dorsoventral axis in Xenopus laevis embryo. *Development* **124**, 2553-60.

Maeno, M., Ong, R. C., Suzuki, A., Ueno, N. and Kung, H. F. (1994). A truncated bone morphogenetic protein 4 receptor alters the fate of ventral mesoderm to dorsal mesoderm: roles of animal pole tissue in the development of ventral mesoderm. *Proc. Natl. Acad. Sci. U S A* **91**, 10260-4.

Masuyama, N., Hanafusa, H., Kusakabe, M., Shibuya, H. and Nishida, E. (1999). Identification of two Smad4 proteins in Xenopus. Their common and distinct properties. *J Biol Chem* **274**, 12163-70.

McMahon, A. P. and Moon, R. T. (1989). Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis. *Cell* **58**, 1075-84.

Miller, J. R. and Moon, R. T. (1996). Signal transduction through beta-catenin and specification of cell fate during embryogenesis. *Genes Dev* **10**, 2527-39.

Miyama, K., Yamada, G., Yamamoto, T. S., Takagi, C., Miyado, K., Sakai, M., Ueno, N. and Shibuya, H. (1999). A BMP-inducible gene, dlx5, regulates osteoblast differentiation and mesoderm induction. *Dev. Biol.* **208**, 123-33.

Moon, R. T. and Kimelman, D. (1998). From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in Xenopus. *Bioessays* **20**, 536-45.

Niehrs, C. (1999). Head in the WNT: the molecular nature of Spemann's head organizer. *Trends Genet* **15**, 314-9.

Nieuwkoop, P. D. and Faber, J. (1967). *A normal table of* Xenopus laevis. Amsterdam: North Holland Publishing Co.

Nishimatsu, S., Suzuki, A., Shoda, A., Murakami, K. and Ueno, N. (1992). Genes for bone morphogenetic proteins are differentially transcribed in early amphibian embryos. *Biochem Biophys Res Commun* **186**, 1487-95.

Nusse, R. and Varmus, H. E. (1992). Wnt genes. *Cell* **69**, 1073-87.

Onichtchouk, D., Gawantka, V., Dosch, R., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C. (1996). The Xvent-2 homeobox gene is part of the BMP-4 signalling pathway controlling [correction of controling] dorsoventral patterning of Xenopus mesoderm. *Development* **122**, 3045-53.

Onichtchouk, D., Glinka, A. and Niehrs, C. (1998). Requirement for Xvent-1 and Xvent-2 gene function in dorsoventral patterning of Xenopus mesoderm. *Development* **125**, 1447-56.

Osada, S. I., Saijoh, Y., Frisch, A., Yeo, C. Y., Adachi, H., Watanabe, M., Whitman, M., Hamada, H. and Wright, C. V. (2000). Activin/nodal responsiveness and asymmetric expression of a Xenopus nodal-related gene converge on a FAST-regulated module in intron 1. *Development* **127**, 2503-14.

Papalopulu, N. and Kintner, C. (1993). Xenopus Distal-less related homeobox genes are expressed in the developing forebrain and are induced by planar signals. *Development* **117**, 961-75.

Papalopulu, N. and Kintner, C. (1996). A Xenopus gene, Xbr-1, defines a novel class of homeobox genes and is expressed in the dorsal ciliary margin of the eye. *Dev. Biol.* **174**, 104-14.

Persson, U., Izumi, H., Souchelnytskyi, S., Itoh, S., Grimsby, S., Engstrom, U., Heldin, C. H., Funa, K. and ten Dijke, P. (1998). The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett* **434**, 83-7.

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**, 707-10.

Piccolo, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-98.

Rastegar, S., Friedle, H., Frommer, G. and Knochel, W. (1999). Transcriptional regulation of Xvent homeobox genes. *Mech Dev* **81**, 139-49.

Sampath, K., Rubinstein, A. L., Cheng, A. M., Liang, J. O., Fekany, K., Solnica-Krezel, L., Korzh, V., Halpern, M. E. and Wright, C. V. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* **395**, 185-9.

Sasai, Y. and De Robertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. *Dev Biol* **182**, 5-20.

Sasai, Y., Lu, B., Steinbeisser, H. and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. *Nature* **376**, 333-6.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer- specific homeobox genes. *Cell* **79**, 779-90.

Satokata, I., Ma, L., Ohshima, H., Bei, M., Woo, I., Nishizawa, K., Maeda, T., Takano, Y., Uchiyama, M., Heaney, S. et al. (2000). Msx2 deficiency in

mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat Genet* **24**, 391-5.

Satokata, I. and Maas, R. (1994). Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development [see comments]. *Nat Genet* **6**, 348-56.

Schmidt, J. E., von Dassow, G. and Kimelman, D. (1996). Regulation of dorsal-ventral patterning: the ventralizing effects of the novel Xenopus homeobox gene Vox. *Development* **122**, 1711-21.

Sive, H. L. (1993). The frog prince-ss: a molecular formula for dorsoventral patterning in Xenopus. *Genes Dev.* **7**, 1-12.

Smith, J. C., Price, B. M., Van Nimmen, K. and Huylebroeck, D. (1990). Identification of a potent Xenopus mesoderm-inducing factor as a homologue of activin A. *Nature* **345**, 729-31.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-40.

Spemann, H. (1931). Über den anteil von implantat und wirtskeim an der orientierung und beschaffenheit der induzierten embryonalanlage. *Wilhelm Roux Arch. EntwMech. Org.* **123**, 389-517.

Spemann, H. and Mangold, H. (1924). Über induktion von embryonalanlagen durch implantation artfremder organisatoren. *Wilhelm Roux Arch. EntwMech. Org.* **100**, 599-638.

Suzuki, A., Kaneko, E., Maeda, J. and Ueno, N. (1997a). Mesoderm induction by BMP-4 and -7 heterodimers. *Biochem. Biophys. Res. Commun.* **232**, 153-6.

Suzuki, A., Shioda, N. and Ueno, N. (1995). Bone morphogenetic protein acts as a ventral mesoderm modifier in early Xenopus embryos. *Dev. Growth. Differ.* **37**, 581-588.

Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo. *Proc. Natl. Acad. Sci. U S A* **91**, 10255-9.

Suzuki, A., Ueno, N. and Hemmati-Brivanlou, A. (1997b). Xenopus msx1 mediates epidermal induction and neural inhibition by BMP4. *Development* **124**, 3037-44.

Sykes, T. G., Rodaway, A. R., Walmsley, M. E. and Patient, R. K. (1998). Suppression of GATA factor activity causes axis duplication in Xenopus. *Development* **125**, 4595-605.

Szebenyi, G. and Fallon, J. F. (1999). Fibroblast growth factors as multifunctional signaling factors. *Int Rev Cytol* **185**, 45-106.

Taira, M., Jamrich, M., Good, P. J. and Dawid, I. B. (1992). The LIM domain-containing homeo box gene Xlim-1 is expressed specifically in the organizer region of Xenopus gastrula embryos. *Genes Dev.* **6**, 356-66.

Takahashi, S., Yokota, C., Takano, K., Tanegashima, K., Onuma, Y., Goto, J. and Asashima, M. (2000). Two novel nodal-related genes initiate early inductive events in Xenopus Nieuwkoop center. *Development* **127**, 5319-29.

Takeda, M., Saito, Y., Sekine, R., Onitsuka, I., Maeda, R. and Maeno, M. (2000). Xenopus msx-1 regulates dorso-ventral axis formation by suppressing the expression of organizer genes. *Comp Biochem Physiol B Biochem Mol Biol* **126**, 157-68.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D. A. (1990). Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. *Cell* **63**, 485-93.

Urist, M. R. (1965). Bone: formation by autoinduction. *Science* **150**, 893-9.

Vainio, S., Karavanova, I., Jowett, A. and Thesleff, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* **75**, 45-58.

Varlet, I., Collignon, J. and Robertson, E. J. (1997). nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development* **124**, 1033-44.

Vogel, A., Roberts-Clarke, D. and Niswander, L. (1995). Effect of FGF on gene expression in chick limb bud cells in vivo and in vitro. *Dev. Biol.* **171**, 507-20.

Walmsley, M. E., Guille, M. J., Bertwistle, D., Smith, J. C., Pizzey, J. A. and Patient, R. K. (1994). Negative control of Xenopus GATA-2 by activin and noggin with eventual expression in precursors of the ventral blood islands. *Development* **120**, 2519-29.

Watabe, T., Kim, S., Candia, A., Rothbacher, U., Hashimoto, C., Inoue, K. and Cho, K. W. (1995). Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between Xenopus and mouse. *Genes Dev.* **9**, 3038-50.

Watanabe, M. and Whitman, M. (1999). FAST-1 is a key maternal effector of mesoderm inducers in the early Xenopus embryo. *Development* **126**, 5621-34.

Weinstein, D. C. and Hemmati-Brivanlou, A. (1999). Neural induction. *Annu Rev Cell Dev Biol* **15**, 411-33.

Wilson, P. A. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376**, 331-3.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. and Wang, E. A. (1988). Novel regulators of bone formation: molecular clones and activities. *Science* **242**, 1528-34.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry, W. L., 3rd, Lee, J. J., Tilghman, S. M., Gumbiner, B. M. and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell* **90**, 181-92.

Zimmerman, L. B., De Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.

Zorn, A. M., Butler, K. and Gurdon, J. B. (1999). Anterior endomesoderm specification in Xenopus by Wnt/beta-catenin and TGF-beta signalling pathways. *Dev Biol* **209**, 282-97.