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Xmsx-1 modifies mesodermal tissue pattern along dorsoventral axis in *Xenopus laevis* embryo

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SUMMARY

This study analyzes the expression and the function of *Xenopus msx-1* (*Xmsx-1*) in embryos, in relation to the ventralizing activity of bone morphogenetic protein-4 (BMP-4). Expression of *Xmsx-1* was increased in UV-treated ventralized embryos and decreased in LiCl-treated dorsalized embryos at the neurula stage (stage 14). Whole-mount in situ hybridization analysis showed that *Xmsx-1* is expressed in marginal zone and animal pole areas, laterally and ventrally, but not dorsally, at mid-gastrula (stage 11) and late-gastrula (stage 13) stages. Injection of *BMP-4* RNA, but not *activin* RNA, induced *Xmsx-1* expression in the dorsal marginal zone at the early gastrula stage (stage 10+), and introduction of a dominant negative form of *BMP-4* receptor RNA suppressed *Xmsx-1* expression in animal cap and ventral marginal zone explants at stage 14. Thus, *Xmsx-1* is a target gene specifically regulated by BMP-4 signaling. Embryos injected with *Xmsx-1* RNA in dorsal blastomeres at the 4-cell stage exhibited a ventralized phenotype, with microcephaly and swollen abdomen. Histological observation and immunostaining revealed that these embryos had a large block of muscle tissue in the dorsal mesodermal area instead of notochord. On the basis of molecular marker analysis, however, the injection of *Xmsx-1* RNA did not induce the expression of α-*globin*, nor reduce cardiac α-*actin* in dorsal marginal zone explants. Furthermore, a significant amount of α-*actin* was induced and α-*globin* was turned off in the ventral marginal zone explants injected with *Xmsx-1*. These results indicated that *Xmsx-1* is a target gene of BMP-4 signaling, but possesses a distinct activity on dorsal-ventral patterning of mesodermal tissues.

Key words: *Xmsx-1* RNA, *Xenopus*, dorsoventral axis, mesoderm, dorsalisation, ventralisation, BMP-4

INTRODUCTION

Mesoderm patterning of *Xenopus laevis* embryos is governed by at least two activities. Dorsalizing activity was first defined by Slack et al. (1987), and many genes have been identified in this category; factors include goosecoid (Cho et al., 1991), noggin (Smith and Harland, 1992), chordin (Sasai et al., 1994), follistatin (Hemmati-Brivanlou et al., 1994) and siamois (Lemaire et al., 1995). Among these factors, noggin, chordin and follistatin also possess neural inducing activity (Smith et al., 1993; Sasai et al., 1995; Hemmati-Brivanlou et al., 1994). In addition, more recently, extensive studies have identified ventralizing factors, namely bone morphogenetic protein-4 (BMP-4) (Dale et al., 1992; Jones et al., 1992), *Xwnt-8* (Christian and Moon, 1993) and genes regulated by BMP-4 signaling (Gawantka et al., 1995; Onichtchouk et al., 1996; Schmidt et al., 1996; Ault et al., 1996; Ladher et al., 1996; Mead et al., 1996). It is suggested that these dorsalizing and ventralizing activities make a gradient along the dorsoventral axis and determine the fate of mesoderm.

BMP-4, a secretory protein belonging to the TGF-β super family, is a key factor in the formation of ventral mesoderm, as shown by its expression pattern (Fainsod et al., 1994; Schmidt et al., 1995), and functional studies utilizing dominant negative receptor (Graff et al., 1994; Suzuki et al., 1994; Maéno et al., 1994a) and antisense RNA (Steinbeisser et al., 1995). We have recently investigated downstream factors of BMP-4 signaling. Activation of ras pathway is essential for the BMP-4-mediated erythropoietic differentiation (Xu et al., 1996), as also shown in activin-mediated and FGF-mediated pathways (Whitman and Melton, 1992; MacNicol et al., 1993). *BMP-4* also regulates *XGATA-2* in ventral mesoderm and animal pole tissue. *XGATA-2* is expressed in both prospective epidermis and ventral areas (Walmsley et al., 1994; Kelley et al., 1994), and this factor functions as a stimulator of epidermis-dependent erythropoietic differentiation (Maéno et al., 1996).

To identify nuclear factors involved in ventral pattern formation, we amplified the DNA fragments with homeobox motif from the cDNA of UV-treated gastrula embryos by polymerase chain reaction. We obtained *msx-1* gene fragments in a certain frequency. *Msx-1* is expressed in various places in a specific time course in developing vertebrate embryo (Robert et al., 1989, 1991; Coelho et al., 1991; Vainio et al., 1993; Liem et al., 1995; Graham et al., 1994; Tureckova et al., 1995). In *Drosophila*, *msh*, which is a homolog gene of *msx-1* and *msx-2*, is expressed in muscle progenitor cells and neuronal cells

(Lord et al., 1995). The above studies suggest that the temporal expression of *msx* controls the subsequent growth and differentiation of immature cells and the patterning of tissue structure and that there is a relationship between *msx-1* expression and BMP-4 signaling (Vainio et al., 1993; Liem et al., 1995; Graham et al., 1994; Chen et al., 1996). Thus, we have attempted to elucidate the function and regulation of *Xenopus msx-1* (*Xmsx-1*) in dorsoventral specification in mesoderm formation, in relation to ventralizing activity of BMP-4 signaling. The present study shows that the expression of *Xmsx-1* is regulated at least in part by BMP-4 signaling in *Xenopus* embryonic cells, but the role of *Xmsx-1* in mesoderm patterning is distinct from that of *BMP-4*.

MATERIALS AND METHODS

UV and LiCl treatment

Xenopus laevis embryos were obtained by artificial insemination after induction of females with 250 i.u. of human chorionic gonadotropin. Developmental stages were designated according to Nieuwkoop and Faber (1967). To obtain ventralized embryos, fertilized eggs were freed of the jelly layers with 2.5% thioglycolic acid (pH 8.1), placed in a quartz-bottomed dish filled with 5% MMR and irradiated with UV light for the designated lengths of times using a UV illuminator (Vilber Lourmat Co. Ltd., 254 nm, 29mW/cm^2). These embryos were incubated in 5% MMR at 18°C to stage 14. To obtain the dorsalized embryos, 32-cell-stage embryos were treated with 0.3 M LiCl in 30% MMR for 30 or 40 minutes, thoroughly washed in 30% MMR and then incubated at 23°C to stage 14.

RNA injection and explants

Xenopus Msx-1 plasmid was generous gift from Dr Ramirez (Mount Sinai Medical School, NY). pSP72 containing whole insert (1.8 kb) was linearized with *Sal*I and capped RNA was synthesized using T7 polymerase according to manufacture's protocol (Ambion). Synthetic RNA of *BMP-4* and a dominant negative form *BMP-4* receptor (*DN-TFR11*) were made as described previously (Maéno et al., 1994a; Suzuki et al., 1994). The two blastomeres of dejellied embryos were injected with these RNAs at 2-cell or 4-cell stage (18.4 nl/embryo) in 3% Ficoll/Steinberg's solution. The embryos were developed in Steinberg's solution up to stage 10+, when animal cap (AP), dorsal marginal zone (DMZ) or ventral marginal zone (VMZ) tissue was excised and further cultured in sterilized Steinberg's solution with 30 μg/ml kanamycin.

Northern blot analysis

Total RNA from whole embryos or explants was extracted by AGPC method (RNA isolation kit, Stratagene), loaded in denatured 1% agarose gel and transferred to N-bond membrane (Amersham). Probes used in this study were as follow; *Xmsx-1*, 1.8 kb *Eco*RI fragment (Su et al., 1991); *α-actin*, 1.2 kb *Bam*HI/*Hin*dIII fragment (Mohun et al., 1984); *α-globin*, 0.8 kb *Pst*I fragment (Sandmeier et al., 1988); *EF-1α*, 0.4 kb *Pst*I/*Sac*I fragment (Krieg et al., 1989). Hybridization was performed in Hybrisol I (Oncor) and the membrane was exposed to Kodak XAR-5 film after washing. The same blot was sequentially hybridized with the different probes to detect each message in the same explant samples.

RT-PCR assay

Complementary DNA was synthesized from 500 ng total RNA extracted from 10-15 explants. Polymerase chain reaction (PCR) was performed in 10 μl solution containing 1 μl of cDNA (10% of the obtained cDNA), 1× Ex-Taq buffer, 0.2 mM each of dNTP, 5 μCi of α-^{32}P -dCTP (3000 Ci/mmol), 350 ng of each primer and 0.25 U of Ex-Taq DNA polymerase (Takara, Tokyo). The PCR program consisted of 94°C for 1 minutes, 55°C for 1.5 minutes and 72°C for 1 minutes (22 cycles). After extenstion step (72°C for 10 minutes), 4 μl of the sample was loaded on 5% polyacrylamide gel. Primers used for PCR were as follows: *Xmsx-1*, 5′-GCA-GGA-ACA-TCA-CAC-AGT-CC-3′ and 5′-GGG-TGG-GCT-CAT-CCT-TCT-3′, *EF-1α*, 5′-CCT-GAA-TCA-CCC-AGG-CCA-GAT-TGG-TG-3′ and 5′-GAG-GGT-AGT-CTG-AGA-AGC-TCT-CCA-CG-3′ (Suzuki et al., 1993).

Whole-mount in situ hybridization and immunostaining

Whole-mount in situ hybridization was performed as described by Harland (1991). N-terminal fragment (*Eco*RI/*Hin*dIII, 440 bp) of *Xmsx-1* was subcloned into pSP72 and digoxigenin-labelled riboprobes were synthesized using Sp6 RNA polymerase. The positive signals were visualized using BM purple (Moors Jr. et al., 1995). Whole-mount immunostaining was performed as described (Klymkowsky and Hanken, 1991) using monoclonal antibodies against N-CAM (4d, from Developmental Studies Hybridoma Bank) and keratan sulphate (MZ-15, gift from Dr F. M. Watt) to stain neural tissues and notochord, respectively. Embryos and explants were fixed in Dent solution (20% dimethyl sulfoxide in methanol), incubated in diluted first antibodies and followed by 1:400 diluted peroxidase-conjugated secondary antibodies. Staining was visualized with diaminobenzidine and H_2O_2.

Histology

The embryos and explants cultured for 2 days (stage 35/36 of control embryo) were fixed in 2% paraformaldehyde/50% MMR solution, dehydrated, embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin and eosin. The appearance of each tissue phenotype in explants was determined morphologically and scored as described by Ariizumi and Asashima (1994).

RESULTS

The expression of *Xmsx-1* in explants and embryos

In an attempt to find a homeobox gene with ventralizing activity, we amplified DNA fragments from unidirectional cDNA library made from stage 10+ UV-treated embryos, with homeobox core sequence (TTT-T(G/T)G-AAC-CAG-AT(C/T)-TTC-AC(T/C)-TG) as a primer and T7 sequence (GCG-CGT-AAT-ACG-ACT-CAC-TAT-A) in plasmid as another primer. From the rescued plasmids derived from the amplified DNA fragments, we found a sequence identical to *Xmsx-1* (formerly called as *Xhox7.1*). Thus, we examined the expression of *Xmsx-1* in the UV- and LiCl-treated embryos in order to assess whether dorsoventral patterning affected *Xmsx-1* expression. As shown in Fig. 1, the *Xmsx-1* expression in

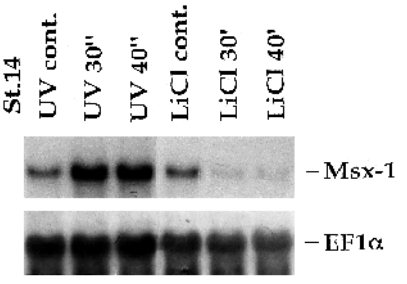

Fig. 1. Expression of *Xmsx-1* in ventralized and dorsalized embryos. Embryos were treated with UV irradiation (30 or 40 seconds) or with LiCl (30 or 40 minutes) as described in Materials and Methods. The RNA was extracted from stage 14 embryos and 10 μg total RNA was electrophoresed in each lane for northern blot analysis. For RNA loading control, the same blot was rehybridized with *EF-1α* probe.

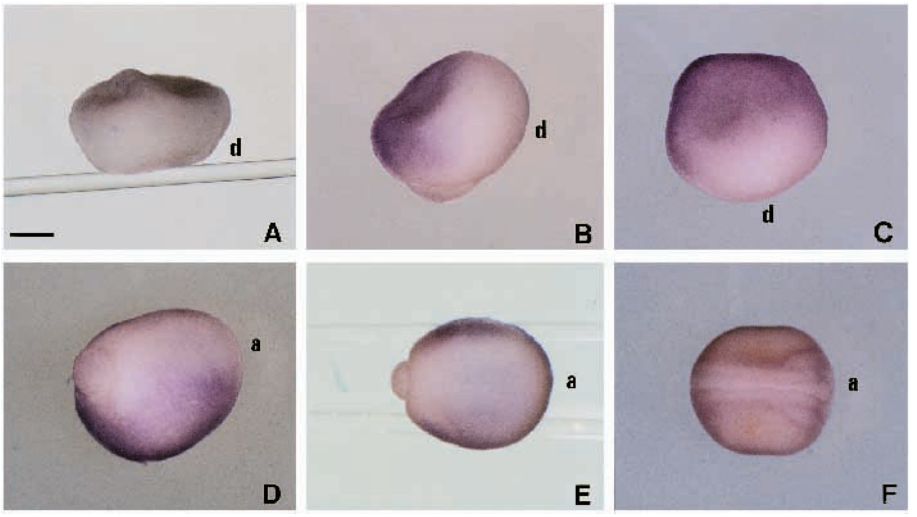

Fig. 2. Expression of *Xmsx-1* in gastrula- and neurula-stage embryos. Whole-mount in situ hybridization was performed using albino embryos. (A) At the early gastrula stage (stage $10^{1/4}$), faint staining was observed in animal hemisphere. (B,C) At mid-gastrula stage (stage 11), staining was detected in marginal zone and animal pole area, laterally and ventrally, but not dorsally. Lateral (B) and animal (C) views. d, dorsal. (D,E) At late gastrula stage (stage 13), message was strongest in dorsal-lateral region and was completely absent in the presumptive neural area. Dorsal-lateral (D) and dorsal (E) views. a, anterior. (F) At neurula stage (stage 15), expression is obviously restricted to the ridges of neural fold and anterior lateral edges of neural region, where the neural crest precursors exist. Bar in A indicates 500 μm.

whole embryos at stage 14 was largely increased in UV-treated embryos and inhibited in LiCl-treated embryos (compared to stage-matched wild-type embryos).

Whole-mount in situ hybridization analysis showed that *Xmsx-1* was just detectable in animal pole area at early gastrula (stage $10^{1/4}$) (Fig. 2A). At mid-gastrula stage (stage 11) (Fig. 2B,C), the positive staining was detected in marginal zone and animal pole area, laterally and ventrally, but not dorsally. The localized expression was much more obvious at late gastrula (stage 13); the message was strongest in dorsal-lateral region, but was completely absent in the presumptive neural area (Fig. 2D,E). At the neurula stage (stage 15), the message was detected in the ridges of neural fold and anterior lateral edges of the neural region, where the neural crest precursors exist (Fig. 2F). These expression patterns suggested that *Xmsx-1* may have a role in dorsoventral specification of the whole embryo at the gastrula stage, in addition to dorsal determination of the neural tube at the neurula stage.

Msx-1 is regulated by BMP-4 signaling

Previous studies have shown that, in mammalian and avian species, BMP-4 signaling governs *msx-1* expression (Vainio et al., 1993; Liem et al., 1995; Graham et al., 1994). Since *BMP-4* is a key factor regulating ventral development in *Xenopus* embryo, we examined whether the expression of *Xmsx-1* is controlled by BMP-4 signaling. *BMP-4* RNA was injected, at the 4-cell stage, into the dorsal marginal zone (DMZ) and expression of *Xmsx-1* was determined in DMZ at stage 10+. As shown in Fig. 3, *Xmsx-1* was prematurely induced by the *BMP-4* RNA injection in DMZ region. The obvious elevation of *Xmsx-1* was only observed when 5 ng of *BMP-4* RNA was introduced. It is concluded that the activation is a direct effect of BMP-4 signaling, since *activin* RNA did not affect the expression of *Xmsx-1* (Fig. 3).

We and others have shown previously that a truncated mutant *BMP-4* receptor RNA (*DN-TFR11*), which exhibits dominant-negative effect on BMP-4 signaling, can alter the fate of ventral tissues to dorsal tissues (Graff et al., 1994; Suzuki et al., 1994; Maéno et al., 1994a). Thus we examined *Xmsx-1* expression when endogenous BMP-4 signaling was blocked in the embryonic tissues by the injection of *DN-TFR11* RNA into animal pole (AP) or VMZ region at 2- or 4-cell

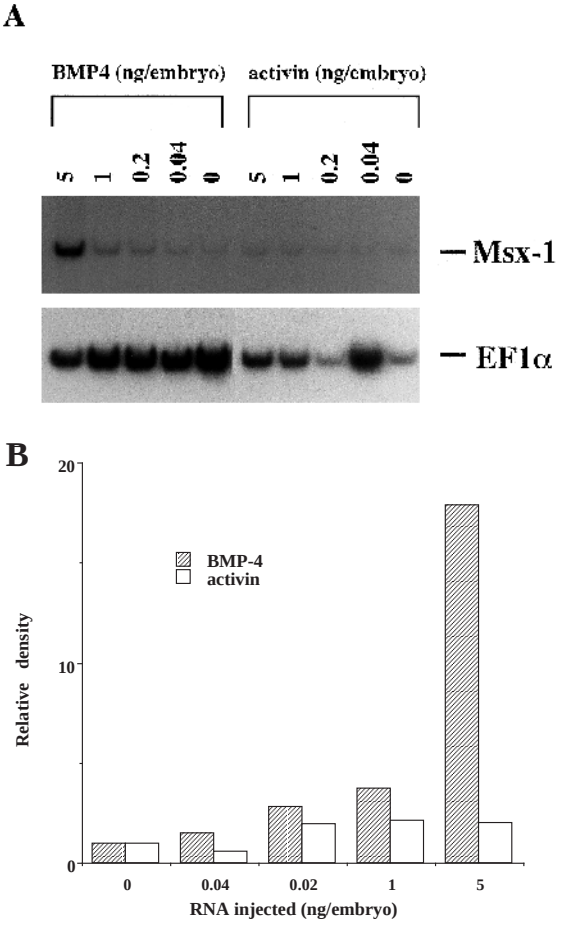

Fig. 3. Expression of *Xmsx-1* is specifically regulated by *BMP-4*. (A) *BMP-4* or *activin* RNA at the concentration indicated was injected into dorsal two blastomeres at 4-cell stage, and dorsal marginal zone (DMZ) tissues were excised at early gastrula (stage 10+). Total RNA from 10-15 explants were subjected to RT-PCR analysis to detect *Xmsx-1* and *EF-1α* mRNAs. (B) The densitometric analysis was performed with a scanning imager (Personal Densitometer, Molecular Dynamics Japan, Tokyo, Japan). The relative density of each sample was normalized by the densitometric values of uninjected DMZ explants. Note that *BMP-4*, but not *activin*, was able to enhance the expression of *Xmsx-1* in a dose-dependent manner.

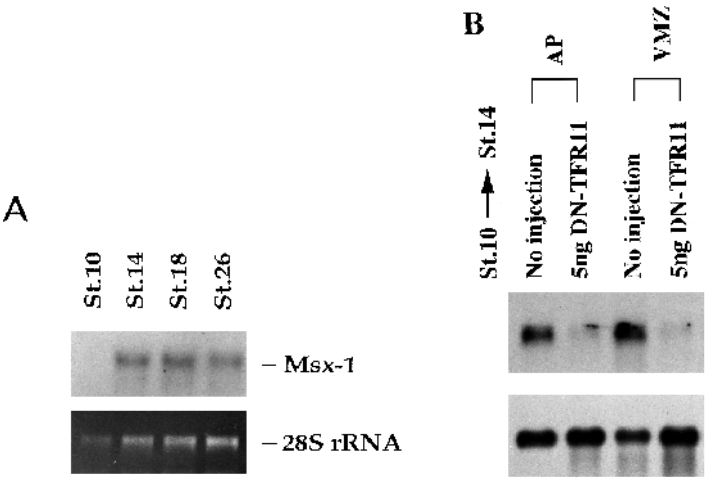

Fig. 4. *DN-TFR11* RNA blocks the expression of *Xmsx-1*. (A) The expression of *Xmsx-1* in staged whole embryos. Northern blot analysis was performed as described in Fig. 1. 7 μg total RNA was loaded in each lane. For RNA loading control, 28S ribosomal RNA stained with ethidium bromide is shown below. (B) Inhibition of *Xmsx-1* expression in explants and embryo injected with *DN-TFR11* RNA. Animal pole area (AP) at 2-cell stage or ventral marginal zone (VMZ) at 4-cell stage were injected with capped *DN-TFR11* RNA (5ng/embryo), and each region was cultured from stage 10+ to stage 14. 2 μg total RNA was electrophoresed in each lane.

stages. The explants of each region excised at stage 10+ early gastrula were cultured until stage 14 neurula. *Xmsx-1* was abundantly expressed at stage 14 (Fig. 4A), and also detected in VMZ and AP explants at this stage (Fig. 4B). In contrast, the expression of *Xmsx-1* was drastically reduced both in AP and VMZ explants injected with *DN-TFR11* RNA (Fig. 4B). From these results, it was confirmed that *Xmsx-1* expression is regulated and maintained by BMP-4 signaling in embryonic cells.

Xmsx-1 ventralizes dorsal structures

To assess the activity of *Xmsx-1* on ventralization, we injected *Xmsx-1* RNA in DMZ area and embryos were allowed to develop until stage 35/36. The effect on dorsal development was dose dependent and no significant effect was observed when 0.2 ng RNA was injected per embryo. As shown in Fig. 5B, embryos injected with 5 ng *Xmsx-1* RNA exhibited microcephaly with smaller eye capsules. The average D. A. I. of *Xmsx-1*-injected embryos was 3.55 (*n*=33). Whole-mount immunostaining with anti-N-CAM antibody (Fig. 5C,D) and notochord-specific antibody (Fig. 5E,F) showed that dorsal tissues were abortive in injected embryos. Most embryos had a trace of notochord in head region (see arrowhead in Fig. 5F). Histological examination clearly showed that the embryos injected with *Xmsx-1* RNA in dorsal blastomeres have a large block of muscle tissue in the dorsal mesodermal area and notochord is absent at the trunk level (Fig. 6).

We also examined the phenotype of DMZ explants isolated at stage 10+. Injected or control explants were subsequently cultured for 2 days, and the explants were examined by histological and immunohistochemical analyses. The control DMZ explants exhibited a typical dorsal phenotype as shown by the appearance of the cement gland (Fig. 7A). In contrast, those

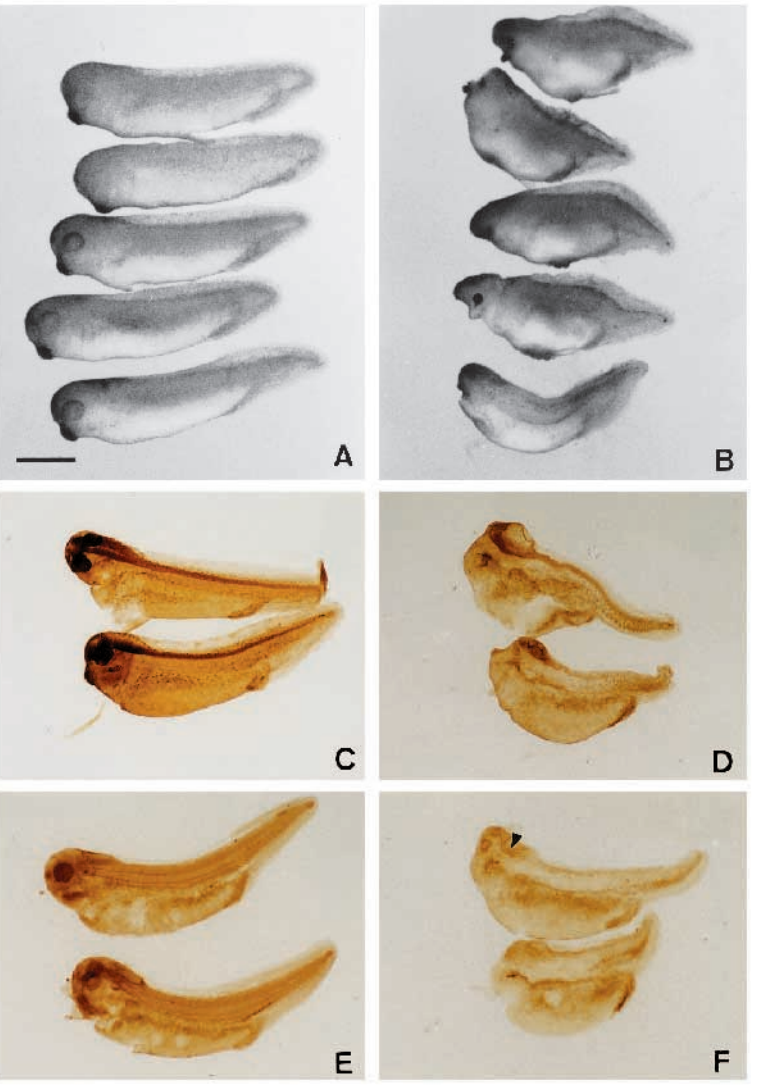

Fig. 5. Morphological views of whole embryos injected with *Xmsx-1* RNA. Dorsal two blastomeres at 4-cell stage were injected with capped *Xmsx-1* RNA (5 ng/embryo), and these embryos (B,D,F) or control uninjected embryos (A,C,E) were allowed to develop to stage 35/36. The embryos injected with *Xmsx-1* exhibit microcephaly with small eye capsules and swollen abdomens. Some embryos were immunostained with anti-N-CAM antibody, 4d (C,D) or with anti-notochord antibody, MZ-15 (E, F), showing significant reduction of both markers in injected embryos (D, F). Arrowhead in F shows a trace of notochord in *Xmsx-1*-injected embryo. Bar in A indicates 1 mm.

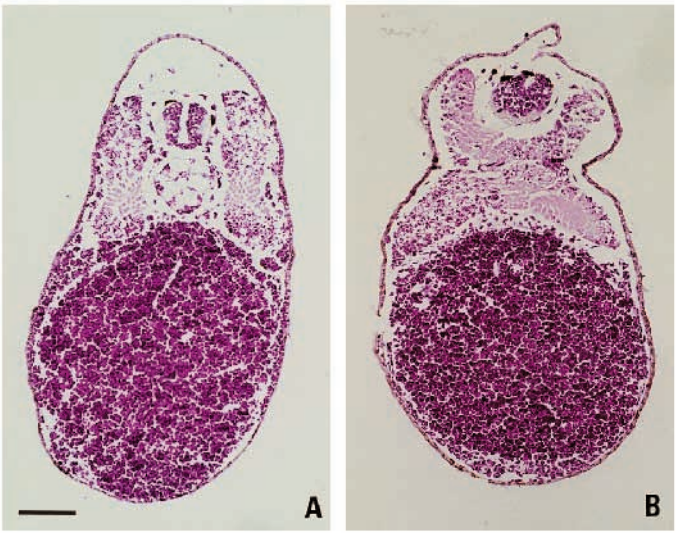

Fig. 6. Histological views of whole embryos injected with *Xmsx-1* RNA. Transverse sections through mid-trunk region of a control uninjected embryo (A) and an embryo injected with *Xmsx-1* (B). The same embryos shown in Fig. 5A and 5B were used for histological examination. A large block of muscle tissue was observed in dorsal mesodermal area of the embryo injected with *Xmsx-1* RNA. Bar in A indicates 100 μm.

explants injected with 5 ng *Xmsx-1* showed a swollen, clear tissue type (Fig. 7B). Histological examination of these explants showed that, while the control DMZ explants contained neural tissue, cement gland, notochord and muscle in most cases, injected explants had mesenchyme instead of dorsal tissues (Figs 7D-F, 8). Although it is clear from the tissue pattern in the injected explants that *Xmsx-1* possesses a ventralizing activity (inhibiting notochord and neural tissue development), we found that muscle, a dorsolateral type tissue, was abundant in the explants (Figs 7D, 8). Staining of these explants with antibodies also demonstrated that neural tissue (Fig. 7C) and notochord (data not shown) were absent after injection of *Xmsx-1*.

Xmsx-1 has an activity distinct from *BMP-4*

We compared the biological activity on mesoderm specification of *Xmsx-1* with that of *BMP-4* . The blastomeres of DMZ, LMZ or VMZ regions were injected with either *BMP-4* RNA or *Xmsx-1* RNA, and each region was explanted and cultured for 2 days. *BMP-4* completely converted dorsal phenotype of the DMZ explants to a ventral phenotype. In both DMZ and LMZ explants, muscle *α-actin* mRNA was diminished and *α-globin* mRNA was induced. In contrast, *Xmsx-1*-injected explants preserved the production of abundant muscle *α–actin* mRNA and did not express any trace of *α-globin* mRNA (Fig. 9). Furthermore, surprisingly, *Xmsx-1* can strongly induce *α-actin* mRNA and completely inhibit *α-globin* mRNA expression in VMZ explants (Fig. 9). These results revealed that, even though

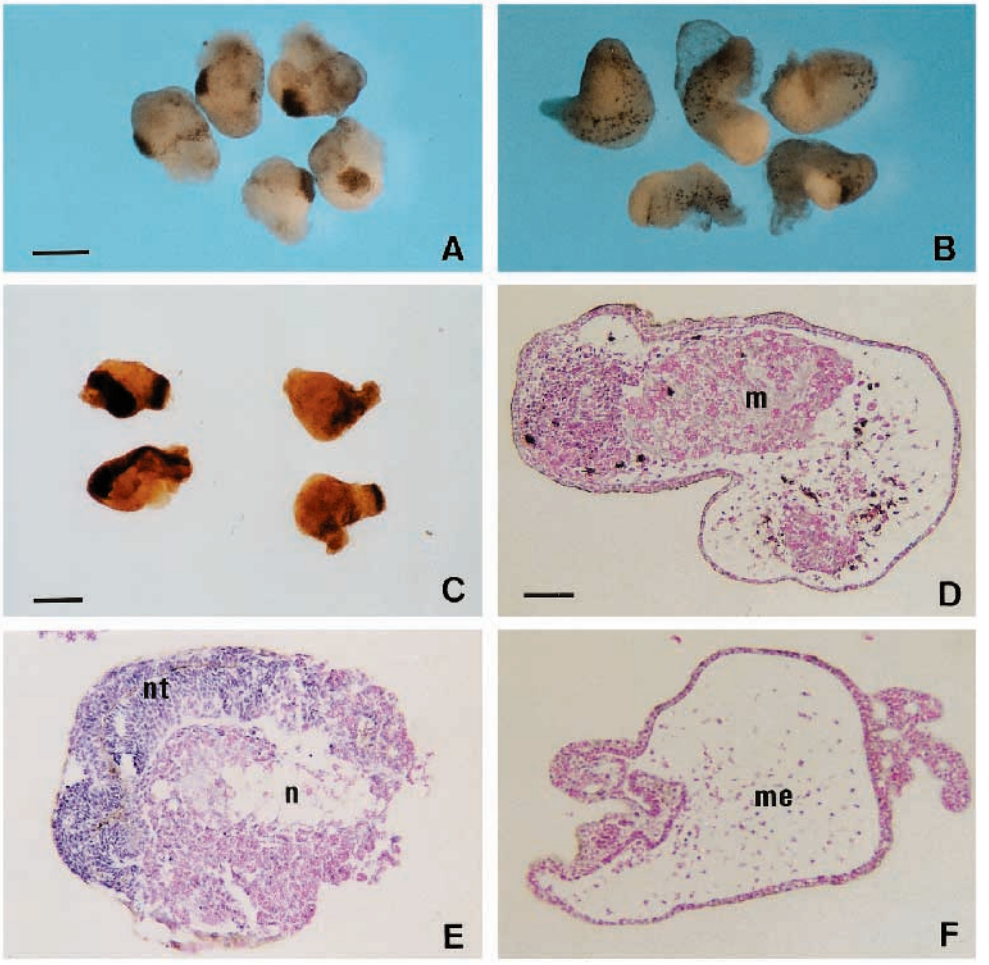

Fig. 7. Morphological and histological views of dorsal marginal zone (DMZ) explants injected with *Xmsx-1* RNA. Dorsal two blastomeres at 4-cell stage were injected with capped *Xmsx-1* RNA (5 ng/embryo) and DMZ tissues of the control (A) and injected (B) embryos were excised at early gastrula (stage 10+) for subsequent culture for 2 days. (C) Control (left) or *Xmsx-1*-injected (right) DMZ explants were immunostained with anti-N-CAM antibody, 4d. Histological observation of control (E) and *Xmsx-1*-injected (D,F) DMZ explants shows that cement gland, neural tissue and notochord were absent, and mesenchymal tissue and muscle were present in the injected explants. Bars in A,C and D indicate 500 μm, 500 μm and 100 μm, respectively. m, muscle; me, mesenchyme; n, notochord; nt, neural tissue.

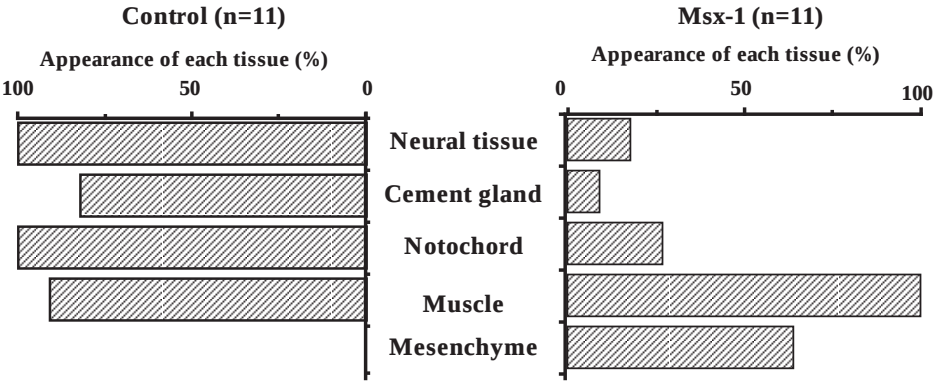

Fig. 8. Summary of tissue types in the dorsal marginal zone explants (DMZ) with or without injection of capped *Xmsx-1* RNA (5 ng/embryo). Serial sections from each explant were prepared and the percentage of explants containing each tissue in total explants (*n*=11) is represented.

expression is regulated by BMP-4 signaling, *Xmsx-1* functions in determination and differentiation of dorsolateral region of mesodermal derivatives including muscle tissue.

We also investigated whether *Xmsx-1* could rescue the dorsalizing effect of *DN-TFR11* on VMZ explants. As shown in previously, injection of high doses of *DN-TFR11* RNA into the ventral blastomeres and subsequent culture of VMZ from stage 10+ abrogated the ventral phenotypes in the explants and led to the appearance of dorsal phenotypes such as notochord, muscle and neural tissue. As expected from the results of Fig. 9, coexpression of *DN-TFR11* and *Xmsx-1* RNAs in the VMZ explants did not recover the *α-globin* expression (data not shown). This indicates that, although *Xmsx-1* is a downstream target gene of BMP-4 signaling that is involved in ventral specification, other factors independent from BMP-4 signaling might be involved in the specific expression and function of *Xmsx-1* during embryonic axis formation.

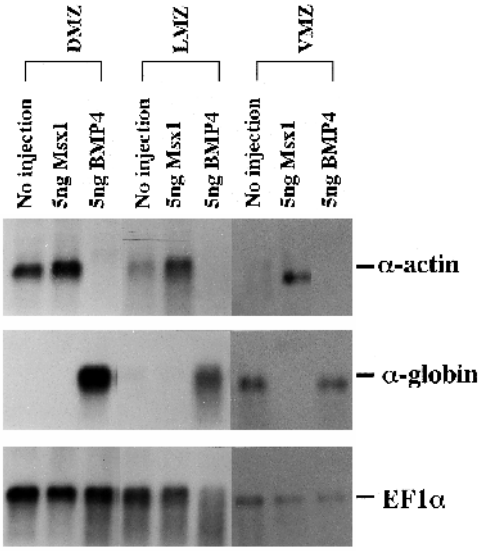

Fig. 9. The distinct ventralizing activities in inducing mesodermal tissues raised by *BMP-4* and *Xmsx-1*. Dorsal, lateral or ventral two blastomeres at 4-cell stage were injected with *Xmsx-1* or *BMP-4* RNA (5 ng /embryo), and dorsal/lateral/ventral marginal zone (DMZ/LMZ/VMZ) tissues were excised at early gastrula (stage 10+) for subsequent culture for 2 days. Total RNA from 10-15 explants were extracted and 2 μg RNA was electrophoresed in each lane for northern blot analysis to detect *α-actin*, *α-globin* and *EF-1α*. Note that *Xmsx-1* induced the expression of *α-actin* and inhibited the expression of *α-globin* in VMZ explants.

DISCUSSION

A novel function of *Xmsx-1* on mesoderm specification

A number of studies have shown that BMP-4, a growth peptide belonging to TGF-β family, is a key factor in the regulation of the pattern of mesoderm formation in *Xenopus* embryo (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Schmidt et al., 1995; Graff et al., 1994; Suzuki et al., 1994; Maéno et al., 1994a; Steinbeisser et al., 1995). The action of BMP-4 in relation to the dorsalizing factors (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995), FGF signaling (Northrop et al., 1995), and activin signaling (Dale et al., 1992; Jones et al., 1992; Suzuki et al., 1995) has been analyzed, and also recent studies have investigated the target factors located downstream of the BMP-4 signal (Gawantka et al., 1995; Onichtchouk et al., 1996; Ladher et al., 1996; Schmidt et al., 1996; Ault et al., 1996; Mead et al., 1996; Xu et al., 1996; Graff et al., 1996; Liu et al., 1996). Our previous study focused on the role of *XGATA-2* in ventral mesoderm formation. *XGATA-2* expression can be induced by the *BMP-4* overexpression in the animal pole area and *XGATA-2* can activate the epidermis-dependent stimulation on the ventral mesoderm to form blood cells. However, the knockout of BMP-4 signal by a dominant-negative receptor (*DN-TFR11*) did not inhibit completely *XGATA-2* expression in animal pole tissue. Thus we concluded that *XGATA-2* is not solely maintained and regulated by BMP-4 signal (Maéno et al., 1996). In contrast to *XGATA-2*, the expression of *Xmsx-1* is drastically decreased by the *DN-TFR11* injection (Fig. 4). In this respect, *Xmsx-1* expression (and possibly function) is dependent on BMP-4 signaling that leads to ventralization of the frog embryo. The correlation of BMP-4 signaling with the expression and the action of *msx-1* has also been suggested by previous studies using different experimental models (Graham et al., 1994; Liem et al., 1995; Tureckova et al., 1995).

Expression of *Xmsx-1* in gastrula and neurula embryos

In agreement with the previous work (Su et al., 1991), whole-mount in situ hybridization showed that *Xmsx-1* is highly expressed in the dorsal region of neural tube and in neural crest cells adjacent to the forebrain and midbrain at the neurula stage. Although in situ analysis indicated that the *Xmsx-1* message is localized in neural cells at neurula stage, our northern blot analysis showed that VMZ explants at the neurula stage also express a significant amount of message (Fig. 4B). Thus, it would be pertinent to conclude that *Xmsx-1* is not solely expressed in neural cells but also in lateral and ventral portions

at this stage. Furthermore, our data emphasized that *Xmsx-1* message was detected in lateral and ventral areas of mid to late gastrula embryos (Fig. 2). This expression pattern suggests a role of *Xmsx-1* in determining dorsoventral pattern of mesoderm derivatives. Especially, the strong expression in dorsolateral areas (shown in Fig. 2D) might reflect an importance of this gene in differentiation of muscle tissue, as is also shown in studies using RNA injection (Figs 6, 9). Further studies using a cytogenetic marker system will be important to prove whether muscle progenitor cells temporally express *Xmsx-1* or not.

Biological activities of *Xmsx-1* in determining mesoderm derivatives

While the present study showed that *Xmsx-1* disturbs the differentiation of notochord and neural tissues, Chen and Solursh (1995) reported recently that the injection of *Xmsx-1* into uncleaved fertilized egg caused axis duplication of dorsal structures including notochord, neural tube and somites. The reason for this discrepancy is unclear. As suggested in our expression study, taken together with other studies on *msx-1* expression (Su et al., 1991; Liem et al., 1995; Tureckova et al., 1995), the function of *Xmsx-1* may be complex and distinct in different cell lineages. In the present study, the ventral cells, if they are injected with *msx-1*, can be dorsalized (blood cells were converted to muscle tissue), and likewise the dorsal cells can be ventralized (notochord was converted to muscle tissue). Therefore, it is speculated that the injection of *Xmsx-1* RNA into fertilized cells reorganized the dorsoventral axis in some injected embryos and secondary axis was formed. The differentiation of muscle tissue from ventral mesoderm in this study might be coincident with this observation.

The most important finding in this study is a difference in biological activities on mesoderm formation between *BMP-4* and *Xmsx-1*. (1) Animal cap explants from a stage 10+ embryo, which was previously injected with *BMP-4* RNA, showed a swollen structure with slight elevation of *α-globin* mRNA (Dale et al., 1992; Maéno et al., 1994a). Thus *BMP-4* is a mesoderm inducer. In contrast, animal cap injected with *Xmsx-1* RNA gave rise to atypical epidermis (data not shown). This indicates that *Xmsx-1* has no activity to induce any mesoderm and may regulate the mesoderm patterning after primary induction of mesoderm. (2) *Xmsx-1* ventralized the dorsal mesodermal structures and mesenchymal cells were obviously induced in DMZ explants in terms of morphological examination. However, it did not induce any trace of *α-globin* mRNA. *BMP-4*, in contrast, could turn on *α-globin* mRNA (Fig. 9), suggesting that *Xmsx-1* does not function in the development of blood cells in the embryo. This phenomenon was also supported by the following result. We have shown previously that *BMP-4* can stimulate the epidermis-dependent erythropoietic activation in combination explants of ventral mesoderm with animal pole tissue (Maéno et al., 1994b; Xu et al., 1996). *Xmsx-1* failed to activate the blood program in this system. Thus, activity stimulated by *BMP-4* can not be replaced by *Xmsx-1* in ectodermal cells. (3) *BMP-4* completely abrogated muscle formation in the DMZ explants based on muscle α-actin expression, but *Xmsx-1* did not. Furthermore, in VMZ explants, α-actin message was turned on and α-globin message was turned off by the injection of *Xmsx-1* RNA. These observations suggest that, although the expression of *Xmsx-1* is regulated by BMP-4 signaling, other factors could be involved in the positive and negative regulation of *Xmsx-1* expression.

Roles of *Xmsx-1* on muscle tissue differentiation

Finally, the inducible effect of *Xmsx-1* on muscle differentiation is inconsistent with previous reports, in which human *msx-1* suppresses *myoD* expression and differentiation of myoblasts to muscle cells (Song et al., 1992; Woloshin et al., 1995). However, we propose that myogenic differentiation in fibroblasts and embryonic mesodermal cells involve distinct mechanisms. As also supposed in myogenic regulation by *twist* (Spicer et al., 1996; Baylies and Bate, 1996), *msx-1* may be necessary in early phases of muscle precursor cells, but not in differentiating myoblasts. Thus, the undifferentiated mesodermal cells may need a transient *msx-1* expression for the determination of these cells to myogenic lineage, but at a later stage, the cells do not need *msx-1* for further differentiation. Experiments to elucidate distinct regulation mechanisms in these two systems are currently being undertaken.

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