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Role of BMP-4 in the Inducing Ability of the Head Organizer in *Xenopus laevis*

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ABSTRACT—BMP-4 has been implicated in the patterning of the Dorsal-Ventral axis of mesoderm and ectoderm. In this study, we describe the posteriorizing effect of BMP-4 on the neural inducing ability of dorsal mesoderm (dorsal lip region) in *Xenopus* gastrulae. Dorsal lip explants dissected from stage 10.25 embryos retained anterior inducing ability when precultured for 6 hrs until sibling embryos reach stage 12. When the dorsal lips from stage 10.25 embryos were treated with a range of BMP-4 concentrations, posterior tissues were induced in adjacent ectoderm in a dose-dependent manner. Thus activin-treated explants able to act as head inducers can also induce posterior structures in the presence of BMP-4. To investigate whether BMP-4 directly affects the inducing ability of dorsal mesoderm, we blocked the BMP-4 signaling pathway by injection of mRNA encoding a truncated form of the BMP-4 receptor (tBR) mRNA. Under these conditions, activin-treated explants induced anterior tissues following BMP-4 treatment. Taken together, these results indicate that BMP-4 may affect the head inducing ability of dorsal mesoderm and confer trunk-tail inducing ability during *Xenopus* gastrulation.

Key words: embryonic axis, dorsal lip, TGF-β, activin, posterior

INTRODUCTION

In 1924, Spemann and Mangold used a heteroplastic transplantation technique to show that the dorsal blastopore lip (dorsal lip) of amphibian gastrula has body axis inducing ability. When transplanted into the ventral blastocoel of another embryo, the dorsal lip was shown to induce a secondary embryo (Spemann and Mangold, 1924). Subsequently, Spemann found that dorsal lip of early amphibian gastrula could induce secondary head structures (head organizer) when transplanted into the blastocoel of another early gastrula, while that of late amphibian gastrula induced trunk-tail structures (trunk-tail organizer) (Spemann, 1931). In addition, Mangold showed that the anterior region of the archenteron roof at the neurula stage was the most potent at inducing head structures, whilst the posterior region of the archenteron roof induced trunk-tail structures (Mangold, 1933). These results suggested that the organizer is critical for embryonic patterning and acts in the two distinct regions of head and trunk-tail.

Ligands belonging to the transforming growth factor β (TGF-β) superfamily have been implicated in many developmental processes. During the blastula stage, activin or Vg-1, which exists maternally, activates a signaling pathway and participates in mesoderm induction throughout the marginal zone (Rebagliati *et al*., 1985; Weeks and Melton, 1989; Asashima *et al*., 1990; Ariizumi and Asashima, 1991; Thomesen and Melton, 1993; Asashima, 1994; Fukui *et al*., 1994; Dyson and Gurdon, 1997). In addition, a number of dorsalizing factor also have been identified, including *noggin, chordin,* and *follistatin* (Smith and Harland, 1992; Smith *et al*., 1993; Hemmati-Brivanlou *et al*., 1994; Sasai *et al*., 1994). These secretory proteins bind to BMP and thereby directly inhibit BMP signaling (Piccolo *et al*., 1996; Zimmerman *et al*., 1996; Fainsod *et al*., 1997). The head inducer, Cerberus is a multiple antagonist of Nodal, Wnt, and BMP-4 (Bouwmeester *et al*., 1996; Piccolo *et al*., 1999). BMP antagonists such as this, which are secreted from the organizer, create a graded distribution of BMP signaling in gastrula ectoderm (Wilson *et al*., 1997).

The central nervous system (CNS) is induced and patterned in ectoderm by ligands secreted from the adjacent and underlying mesoderm (reviewed in Sasai and De Robertis, 1997; Asashima *et al*., 2000; Games and Sive, 2000). Two models how A-P axis patterning of neuroectoderm is

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established. In the first model, known as the two-inducer model, anterior and posterior neural tissues are induced via different signals. The ratio of the two inducing factors would determine the A-P axis pattern of the CNS tissues (Tiedemann, 1959; Saxén and Toivonen, 1961). The second model was suggested by Nieuwkoop (1952), and is known as the two-step model (reviewed in Nieuwkoop, 1952a, b). In this model, two steps are proposed to induce CNS patterning along the complete A-P axis. The initial neural inducing signals specify anterior neural tissues, such as forebrain. Subsequently, anterior neural tissues are specified to more posterior neural tissues such as hindbrain and spinal cord via a second later set of signals. The first inducing step is called activation; the second caudalizing step is called transformation. A number of molecules have been identified, which participate in the activation and transformation processes of the two-step model. The anterior neural tissues can be induced by dorsalizing factors, including *noggin, chordin,* and *follistatin* (Lamb *et al*., 1993; Hemmati-Brivanlou *et al*., 1994; Sasai *et al*., 1995). On the other hand, fibroblast growth factor (FGF), retinoic acid (RA), and Xwnt, all secreted factors, are known to caudalize anterior neural tissues thereby acting as transformation signals (Durston *et al*., 1989; Cox and Hemmati-Bribanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; McDrew *et al*., 1995; Papalopulu and Kintner, 1996; Blumberg, 1997; Asashima *et al*., 1999; Games and Sive, 2001). In addition, the wnt/β-catenin and TGF-β signaling pathways cooperate to specify the anterior endomesoderm (Zone *et al*., 1999). Furthermore, the caudalization of anterior neural tissues by bFGF was inhibited in explants expressing the dominant negative Xwnt-8 protein (McDrew *et al*., 1997), and neural induction induced by chordin was inhibited by dominant negative FGF receptor (Sasai *et al*., 1996). These results indicate that lateral mesoderm is implicated in posterior patterning. Despite this, no studies have directly addressed A-P axis patterning of lateral mesoderm during gastrulation (reviewed in Games and Sive, 2000).

It is known that BMP-4 is involved in many developmental processes. It was demonstrated previously that BMP expressed at the ventral marginal zone during the early gastrula stage can induce ventral mesoderm, and pattern the dorsal-ventral axis of the ectoderm and mesoderm (Dale *et al*., 1992; Jones *et al*., 1992; Wilson *et al*., 1997). BMP-4 is also involved in patterning among epidermis, cement gland, and neural tissues (Wilson *et al*., 1997). In light of these observations, experimentation is needed to investigate the effect of BMP-4 on the inductive capacity of dorsal mesoderm during gastrulation.

In this study, we describe the posteriorizing effect of BMP-4 on the neural inducing ability of dorsal mesoderm (dorsal lip region) in *Xenopus* gastrulae in an *in vitro* system. Firstly, we show that complete A-P axis patterning requires marginal zone cells in addition to dorsal mesoderm, which has neural inducing ability. Secondly, we show that the inducing ability of dorsal lips (dorsal mesoderm) dissected from stage 10.25 embryos were altered by treatment of BMP-4. The activin-treated explants, which normally act as head inducers also induced posterior structures in the presence of BMP-4. Thirdly, the affect of BMP-4 on the inducing ability of dorsal mesoderm was tested by injecting the tissue with a truncated form of the BMP-4 receptor (tBR). This work implicates BMP-4 in shifting the head inducing ability of dorsal mesoderm to a posterior inducing ability.

MATERIALS AND METHODS

Xenopus embryos

Xenopus laevis embryos were obtained from adult male and female frogs injected with 600 units of human chorionic gonadotrophin (Gestron, Denka Seiyaku CO. Kawasaki Japan). Fertilized eggs were dejellied by treatment with 4.5% cysteine hydrochloride (Wako, Japan) (pH 7.6) in Steinberg's solution (SS; 58.00 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 3.00 mM hydroxyethylpiperazinyl ethanesulfonic acid, and 100 mg/L kanamycin sulfate, pH 7.4) and washing in sterile SS (pH 7.4). Staging of embryos was performed according to Neiuwkoop and Faber (1956).

Factors

Human recombinant activin was dissolved at 300 ng/ml in SS containing 0.1% Bovine Serum Albumin (BSA: Sigma Chemical CO., St Louis, MO, USA) to avoid adsorption of activin to the plastic surfaces. Recombinant Human Bone Morphogenetic Protein-4 (BMP-4: R&D systems Inc.) was dissolved at various concentrations from 10 ng/ml to 300 ng/ml in SS containing 0.1% BSA.

Manipulation and Culture

The vitelline membrane was manually removed with fine forceps under a stereomicroscope. All operations were carried out under sterile conditions.

Whole embryo assay

Anterior endoderm and ectoderm or dorsal posterior regions (0.4×0.5 mm) were dissected from stage 11 or 12 embryos. We named anterior endoderm and ectoderm as anterior explants, and dorsal posterior regions as posterior explants. The posterior explants were combined with ectodermal explants (0.7×0.7 mm) dissected from late blastulae. Dorsal lips (0.4×0.4 mm) dissected from stage 10.25 embryos were combined with ectodermal explants immediately, or after preculture, for 6 hrs. Control ectoderm explants (0.7×0.7 mm) were dissected from stage 9 embryos cultured alone (Fig. 1A).

Stage 10.25 dorsal lips (0.4×0.4 mm) explants were wrapped in the ectodermal explants containing VMZ cells, which were dissected from stage 10.25 embryos. Ectodermal explants containing VMZ cells cultured alone were used as controls (Fig. 1A).

BMP-4-treatment for dorsal lip explants

Dorsal lips (0.4×0.4 mm) from stage 10.25 embryos were treated with a range of BMP-4 concentrations for 6 hr. Following the BMP-4-treatment, dorsal lips were cultured alone or sandwiched between two sheets of ectodermal explants dissected from stage 9 embryos. As controls, ectodermal explants without additional factors were cultured alone or sandwiched between non-treated ectodermal explants (Fig. 1B).

Activin and BMP-4 treatment for ectodermal explants

The ectodermal explants (0.4×0.4 mm) dissected from stage 9 embryos were treated with 300 ng/ml of activin. The activin-treated

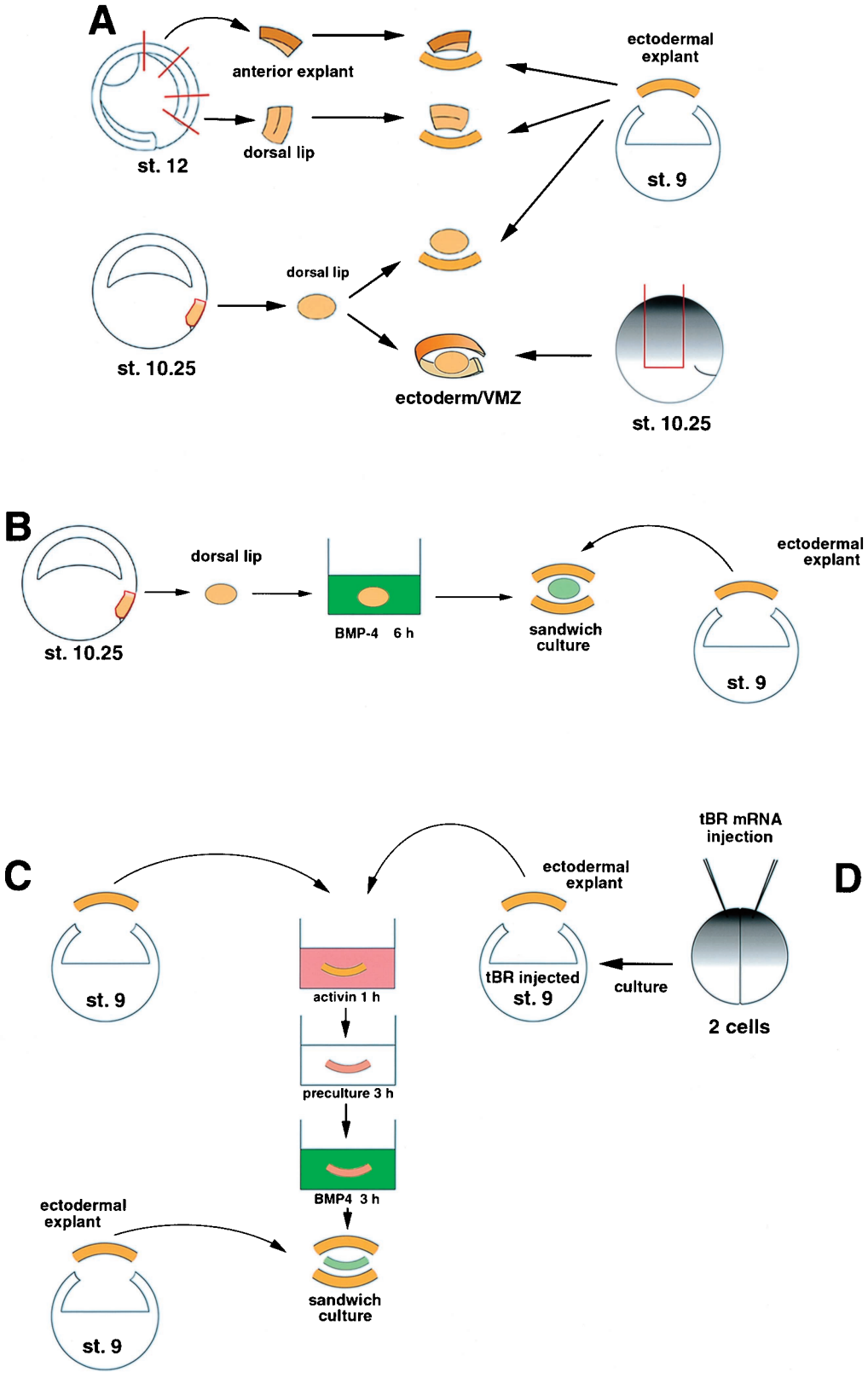


Fig. 1. Schematic diagrams of the experimental procedures (See materials and methods).
(A) Analyses of the inducing ability of the dorsal lip region.
(B) BMP-4 treatment of dorsal lips from stage 10.25 embryos.
(C) Activin and BMP-4 treatment of uninjected ectodermal explants.
(D) Activin and BMP-4 treatment of tBR mRNA injected ectodermal explants.

explants were precultured for 3 hrs. After preculturing, the explants were treated with the range of BMP-4 concentrations for 3 hrs. These activin and BMP-4 treated explants were cultured alone or sandwiched between two sheets of ectodermal explant (Fig. 1C).

Embryos were injected with 200 pg of tBR mRNA into the animal pole at the two-cell stage and cultured until stage 9. The ectodermal explants (0.4×0.4 mm) were dissected from these embryos and treated with 300 ng/ml of activin for 1 hr. After preculturing, the activin-treated explants were treated with various concentration of BMP-4 for 3 hr. These activin and BMP-4 treated explants were cultured alone or sandwiched between two sheets of non-treated ectodermal explant (Fig. 1D).

These explants were cultured in SS containing 0.1% BSA at 20°C.

Microinjection and *In vitro* transcription

Microinjection was performed in SS containing 5% Ficoll. Capped mRNA was synthesized using a mMESSAGE mMACHINE kit (Ambion). Unincorporated ribonucleotide was removed using the RNeasy Mini kit (QIAGEN). The pSP64T-mTRFII-45 del21 (tBR) (Suzuki *et al*., 1994) was linearized with EcoR1 and transcribed with SP6 RNA polymerase.

RT-PCR analysis

Total RNA was extracted from explants by ISOGEN (NIPPON GENE), and 1 μg of total RNA was used as a template to generate first-strand cDNA using Superscript II reverse transcriptase (GIBCO, BRL). EX-Taq DNA polymerase (Takara, Japan) was used in subsequent PCR analyses. The PCR products were electrophoresed on a 1.5% agarose gel (IWAIKAGAKU, Japan) and stained with 10 μg/ml ethidium bromide. The following primer sets were used: *ornithine decarboxylase* (ODC) (Osborne *et al*., 1991), *noggin* (*nog*) and *Xnot* are described in *Xenopus* Molecular Marker Resource (XMMR: http://cbrmed.ucalgary. ca/pvize/html/WWW/welcome.html), *AG-1*, *otx-2*, *en-2*, *Krox-20*, *HoxB9*, *Xbrachyury* (*Xbra*), *muscle-specific actin* (*ms-actin*), *goosecoid* (*gsc*), *cerberus* (*cer*), (Fukui *et al*., 2000), *NCAM* (Kintner and Melton, 1987), *nrp-1* (forward, 5'-GGGTTTCTTGGAACAAGC-3'; reverse, 5'-ACTGTGCAGGAACACAAG-3'), and *chordin* (*chd*) (Sasai *et al*., 1994). Detection of ODC shows comparable amounts of total RNA in each sample.

Histology

The explants were fixed in Bouin's solution (75 ml saturated picric acid, 25 ml formalin, and 5 ml glacial acetic acid) for 3 hrs at room temperature. The samples were then dehydrated through a graded series of ethanol (70%, 90%, 99.5%, and 100%) for 15 min each then infiltrated in Lemosol (Wako Japan) for 20 min, before embedding in paraffin (Histprep 548, Wako Inc., Japan). The samples were sectioned serially at 6 μm and stained with hematoxylin and eosin.

RESULTS

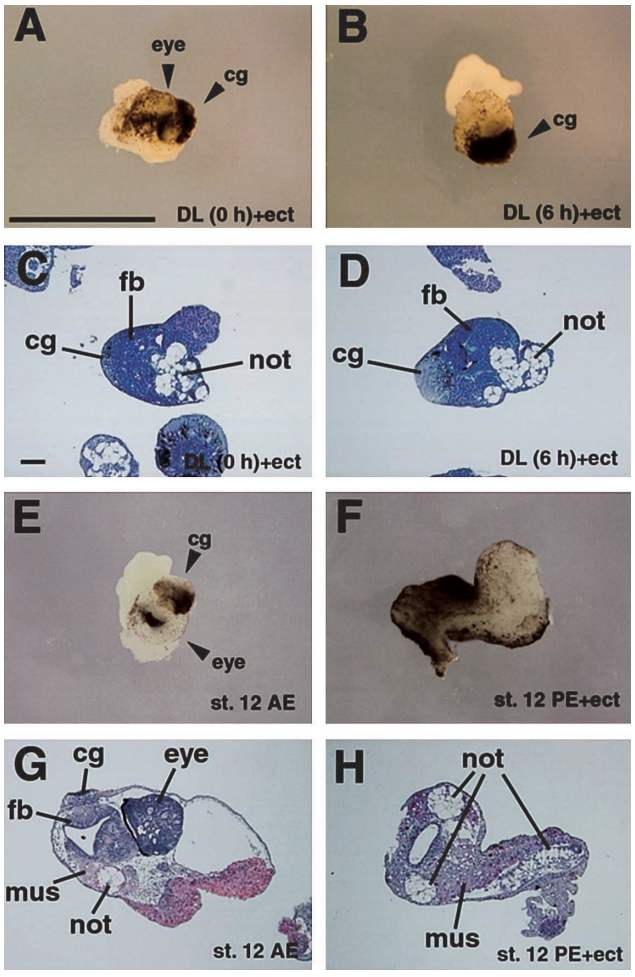
Dorsal lips from early gastrulae can act as a head organizer even after 6 hrs.

It is known that A-P axis patterning is determined before neural induction. However, what determines this patterning is unclear. To address this question, we initially investigated the inducing ability of dorsal lips explanted at different stages of gastrulae development. Dorsal lips explanted at stage 10.25, and dorsal posterior region (posterior explants) explanted at stage 11, or 12 were immediately combined with ectodermal explants dissected from stage 9 embryos. Anterior endodermal mass and ectoderm (anterior explants) were dissected from stage 11 or 12 embryos. Ectodermal explants dissected from stage 9 embryos were cultured alone as controls, without additional factors (as shown in Fig. 1A). To assess tissue differentiation, histological analysis was performed at stage 42. In addition, RT-PCR analysis was performed when sibling embryos reached stage 20 to monitor the expression of region specific neural marker genes. As shown in Fig. 2, dorsal lips dissected from stage 10.25 embryos induced anterior structures in the adjacent ectoderm, such as cement gland and forebrain (Fig. 2A, C). The cement gland marker *AG-1*, forebrain marker *otx-2*, and midbrain-hindbrain boundary marker *en-2* were detected by RT-PCR analysis (Fig. 2I, lane 2). In contrast, posterior explants from stage 11 embryos induced head and trunk-tail structures. The expression of all region specific neural markers, *otx-2*, *en-2*, *Krox-20*, and *HoxB9* could be detected by RT-PCR analysis (Fig. 2I, lane 6). In addition, posterior explants from stage 12 embryos induced posterior structures such as spinal cord (Fig. 2F, H). These explants expressed the hindbrain marker *Krox-20* and the spinal cord marker *HoxB9* (Fig. 2I, lane 4). On the other hand, anterior explants dissected from stage 12 embryos differentiated into anterior structures (Fig. 2E, G), and the histological examination revealed the presence of cement gland and forebrain (Fig. 2G). In the stage 11 embryos, the anterior explants differentiated into atypical epidermis (data not shown). Interestingly, RT-PCR analysis detected the expression of anterior neural markers, *otx-2* and *en-2* in anterior explants from both stages (Fig. 2I, lane5, 7). Non-treated ectodermal explants expressed all of the marker genes tested (Fig. 2I, lane 1) and differentiated into atypical epidermis (data not shown). This result thus indicates that the dorsal lip region of stage 10.25 embryos have anterior inducing ability, however, as gastrulation proceeds, they can also induce trunk-tail structures.

We then examined whether dorsal lips from stage 10.25 embryos possess head inducing ability when precultured for 6 hrs until sibling embryos reach stage 12. After preculturing, dorsal lips were combined with ectodermal explants from stage 9 embryos (Fig. 1A). It was found that the dorsal lips precultured for 6 hrs induced head structures (Fig. 2B, D), and that anterior tissues such as forebrain and cement gland were observed histologically immediately following combination with the ectodermal explants (Fig. 2C, D). No trunk-tail tissues were observed (Fig. 2D). RT-PCR analysis detected expression of the anterior neural markers, *AG-1* and *otx-2* (Fig. 2I, lane 3). These observations indicate that the head inducing ability of the dorsal lip region at embryonic stage 10.25 is unaffected by culturing time.

Dorsal lips from early gastrulae act as both a head and trunk-tail inducer when conjugated with ectoderm containing VMZ cells

In light of the previous experiments, we next examined whether dorsal lips from early gastrulae could induce anterior and posterior structures when conjugated with ectoder-



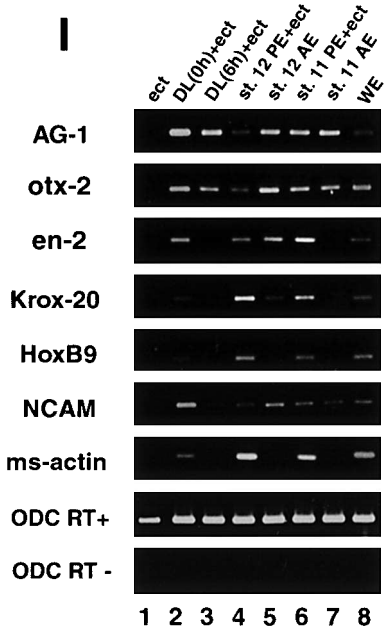
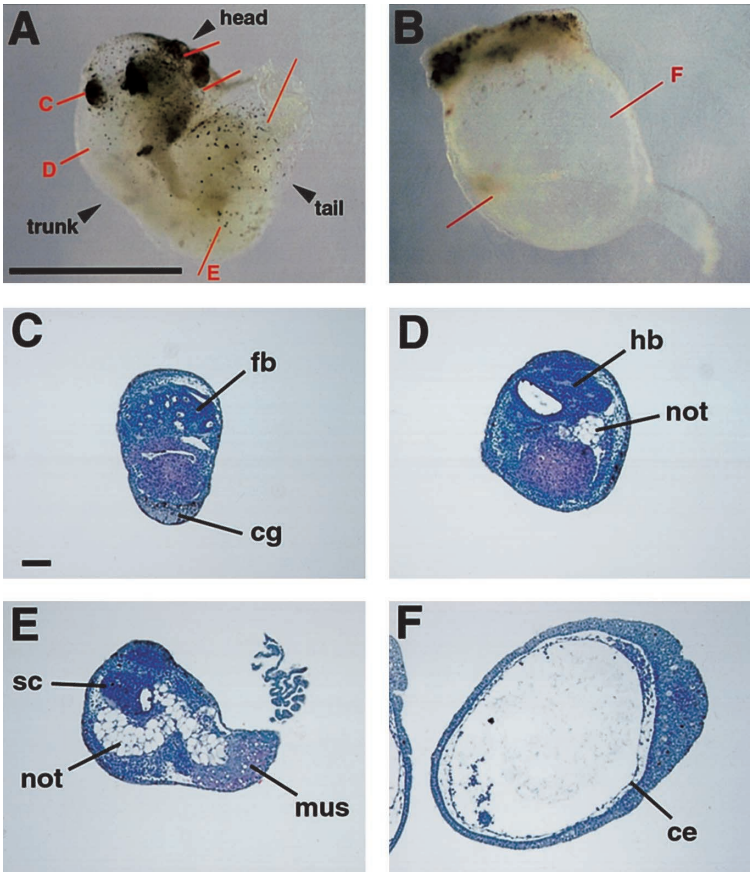


Fig. 2. Dorsal lips from early gastrulae act as a head inducer after 6 hrs. External view (A, B, E, F) and histological section (C, D, G, H) of the explants at stage 42. Dorsal lip explants from stage 10.25 without preculturing (A, C). The anterior structures, forebrain and cement gland were observed (C). Dorsal lip explants from stage 10.25 embryos, precultured for 6 hrs (B, D). The cement gland and forebrain were observed (D). Note that dorsal lip from stage 10.25 embryos had head inducing ability 6 hrs after dissection. Anterior explants from stage 12 embryos (E, G) differentiated into anterior structures. The forebrain, eye, and cement gland were observed (G). Posterior explants from stage 12 embryos (F, H) differentiated into posterior structures including spinal cord, muscle, and notochord (H). cg; cement gland, fb; forebrain, mus; muscle, not; notochord. Scale bar represents 1 mm (A) or 100 μm (C).

(I) RT-PCR analysis of the following marker genes was performed: cement gland *XAG-1*, forebrain *otx-2*, midbrain-hindbrain boundary *en-2*, hindbrain *Krox-20*, spinal cord *HoxB9*, pan-neural *NCAM*, mesodermal *ms-actin*. Total RNA was extracted from the explants cultured to stage 20. Detection of ODC acts as an internal control. ODC RT- indicates experiments without reverse transcriptase. The dorsal lip explants immediately combined with ectodermal explant expressed theanterior neural markers, *otx-2* and *en-2* (lane 2). The dorsal lip explants precultured for 6 hr expressed anterior neural markers, *AG-1* and *otx-2* (lane 3). The posterior explants from stage 12 embryos expressed posterior neural markers, *Krox-20* and *HoxB9* (lane 4). Anterior explants from stage 11 or 12 embryos expressed anterior markers, *AG-1*, *otx-2*, and *en-2* (lane 5, 7). The posterior explants from stage 11 expressed all regional-specific neural markers, *otx-2*, *en-2*, *Krox-20*, and *HoxB9* (lane 6). Non-treated ectodermal explants expressed any markers (lane 1). AE; anterior explant, DL; dorsal lip, ect; ectodermal explant, PE; posterior explant, WE; whole embryo.

mal explants containing ventral marginal zone (VMZ) cells dissected from early gastrulae. Ectodermal explants containing VMZ cells were cultured alone as controls (Fig. 1A). The result was an induction of both head and trunk-tail structures along the A-P axis (Fig. 3A).Forebrain (Fig. 3C), hindbrain (Fig. 3D), and spinal cord (Fig. 3E) were observed



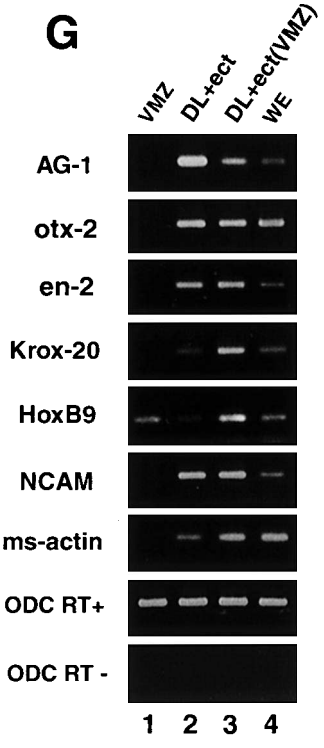


Fig. 3. Dorsal lips from stage 10.25 embryos induced head and trunk-tail structures when combined with ectodermal explants containing VMZ cells.
External view (A, B) and histological section (C-F) of the explants at stage 42.
(A) Head, trunk, and tail structures were observed in the dorsal lip explants combined with ectoderm containing VMZ.
(B) The bulge was observed in the VMZ explant.
(C-D) Histological section of A; the approximate position of the section is indicated by red lines. Forebrain (C), hindbrain (D), and spinal cord (E) were observed.
(F) Histological section of B. Non-axial mesoderm was observed. ce; coelomic epidermis, hb; hindbrain, sc; spinal cord. Scale bar represents 1 mm (A) or 100 μm (C).
(G) RT-PCR analysis of explants was performed at stage 20. The dorsal lips combined with ectoderm containing VMZ cells expressed all regional-specific neural markers, *otx-2*, *en-2*, *Krox-20*, and *HoxB9* (lane 3), whereas the dorsal lips combined with ectoderm only expressed anterior neural markers, *otx-2* and *en-2* (lane 2).

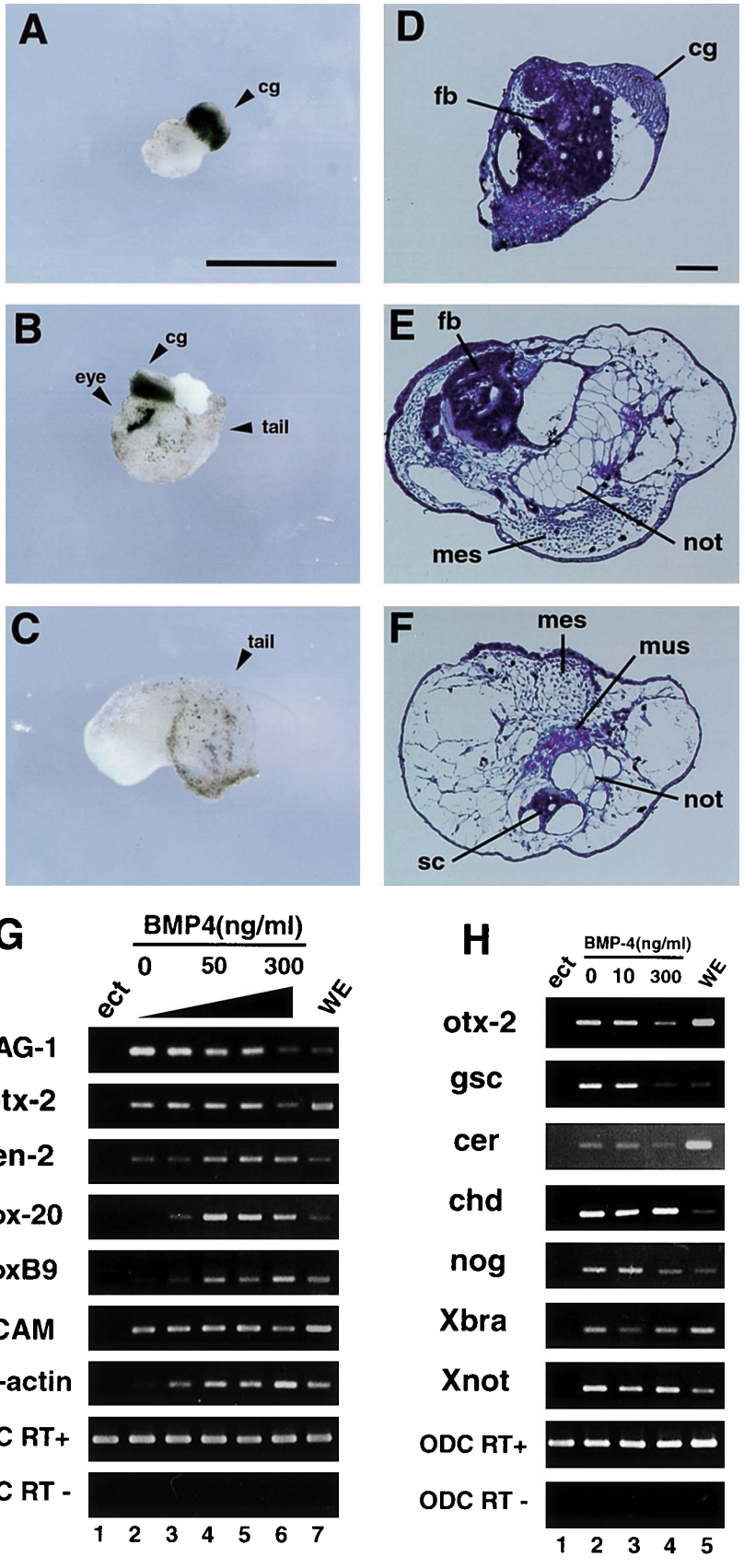


Fig. 4. Dorsal lips from early gastrulae showed a change in inducing ability following BMP-4-treatment.

External view (A-C) and histological section (D-F) of the sandwich explants at stage 42. Sandwich-explants of dorsal lips: non-treated (A, D), treated with 10 ng/ml BMP-4 (B, E), or 100 ng/ml of BMP-4 (C, F). Head-structures, forebrain and cement gland were observed in untreated explants (A, D). At the middle range of BMP-4 concentrations, head and truck-tail structures were observed (B, E). At low concentrations, trunk-tail structures could be observed (C, F). mes; mesenchyme. Scale bar represents 1 mm (A) or 100 μm (D).

(G) RT-PCR analysis was performed on sandwich explants cultured until sibling embryos reached stage 20. At low concentration of BMP-4, anterior neural markers, *otx-2* and *en-2* were expressed (lane 2, 3). In the middle concentration range, posterior neural markers, *Krox-20* and *HoxB9* were upregulated (lane 4). All regional neural markers, *otx-2*, *en-2*, *Krox-20*, and *HoxB9* were detected (lane 4, 5). At high concentrations of BMP-4, anterior neural markers were attenuated and posterior neural markers were expressed (lane 6).

(H) RT-PCR analyses of the following genes was performed on dorsal lips cultured alone until sibling embryos reached stage 12.5: head organizer marker *otx-2*, *cer*, and *gsc*, neural inducer *chd* and *nog*, pan-mesodermal marker *Xbra*, and notochord marker *Xnot*. Total RNA was extracted at stage 12.5. Head organizer markers, *otx-2* and *gsc*, were suppressed by BMP-4 treatment (lane 4). Other markers were not affected (lane 2-4). Non-treated ectodermal explants expressed any markers (lane 1).

by histology. The positional neural markers, *otx-2*, *en-2*, *Krox-20*, and *HoxB9* were detected by RT-PCR at stage 20 (Fig. 3G, lane 3). In contrast, dorsal lips from early gastrulae conjugated with ectodermal explants not containing VMZ cells expressed anterior markers, *otx-2* and *en-2* (Fig. 3G, lane 2). When VMZ cells were cultured alone, non-axial mesoderm could be observed (Fig. 3B, F). These results suggest that dorsal lips from stage 10.25 embryos can induce both head and trunk-tail structures when conjugated with ectoderm containing VMZ cells.

BMP-4 can affect the inducing ability of dorsal lips dissected from stage 10.25 embryos.

From the results thus far, it was predicted that signals released from VMZ cells were important to pattern the complete A-P axis. BMP-4 is a candidate caudalizing signal because it is expressed at the VMZ in early gastrulae and excluded from the organizer region (Dale *et al*., 1992; Jones *et al*., 1992). In addition, BMP-4 is involved in mesodermal and endodermal patterning (Wilson *et al*., 1997). However, there are no reports as to the effect of BMP on the inducing ability of dorsal mesoderm (dorsal lip region) during gastrulation. We addressed this question (Fig. 1B, see Material and Methods). The sandwich-explants of dorsal lips without BMP-4-treatment differentiated into head-structures (Fig. 4A, D). By histology, the cement gland and forebrain could be observed (Fig. 4D). When BMP-4 was applied in the middle concentration range, head and truck-tail structures such as cement gland, forebrain, hindbrain, and spinal cord were observed (Fig. 4B, E). At high concentrations, trunk-tail structures such as spinal cord, muscle, and notochord could be observed (Fig. 4C, F). RT-PCR analysis was performed at stage 20 to detect the expression of regional specific neural marker genes (Fig. 4G). At low BMP-4 concentrations, the anterior neural markers, *otx-2* and *en-2* were expressed (Fig. 4G, lane 2, 3). In the middle of the concentration range, all positional markers tested were expressed, including *otx-2*, *en-2*, *Krox-20*, and *HoxB9* (Fig. 4G, lane 4, 5). At high concentrations of BMP-4, posterior neural markers, *Krox-20* and *HoxB-9* were expressed (Fig. 4G, lane 6). Control explants expressed any marker gene (Fig. 4G, lane 1). The molecular marker analysis was consistent with the histological data, and indicated that BMP-4 may affect the inducing ability of dorsal lips from stage 10.25 embryos to confer trunk-tail inducing ability in a dose dependent manner (Table 1).

Table 1. Inducing ability of dorsal lip explants treated with BMP-4.

	BMP-4 (ng/ml, 3h)	0	10	50	100
	No. of specimens	25	17	22	17
head	cement gland	20(80)	17(100)	16(73)	10(59)
	forebrain	21(84)	15(88)	11(50)	3(18)
	eye	14(68)	7(41)	6(27)	1(6)
trunk	hindbrain	8(32)	12(71)	10(45)	1(6)
tail	spinal cord	6(24)	10(59)	9(41)	5(29)
	mesenchyme	11(44)	8(47)	20(91)	17(100)
	coelomic epidermis	2(8)	2(12)	3(14)	10(59)
	epidermis	22(88)	16(94)	21(95)	16(94)
	notochord	25(100)	17(100)	22(100)	16(94)
	muscle	23(92)	16(94)	21(95)	16(94)
	endodermal masses	19(76)	17(100)	18(82)	10(59)
	atypical epidermis	0(0)	1(6)	1(5)	5(29)

Figure in parentheses indicate the percentage of the number of specimens.

BMP-4 has a qualitative effect on dorsal lips from stage 10.25 embryos without changing their differentiation ability.

To further investigate the effect of BMP-4, we performed an isolation culture of dorsal lips treated with BMP-4 to detect the expression of organizer marker genes or to observe morphological changes. Dorsal lips dissected from stage 10.25 embryos were treated immediately with various concentrations of BMP-4 for 6 hrs. After BMP-4-treatment, dorsal lips were cultured alone until sibling embryos reached stage 12.5. RT-PCR analysis revealed that the head organizer markers, *otx-2* and *gsc*, were suppressed by BMP-4 treatment (Fig. 4H, lane 4). This result agrees with recent data from another group (Yao and Kessler, 2001). The expression of neural inducers *chd* and *nog* was unaffected by BMP-4-treatment, suggesting that the neural inducing ability of dorsal lips is unaffected by BMP-4-treatment. Other markers such as the pan-mesodermal marker *Xbra*, another head organizer marker *cer*, and notochord marker *Xnot* were unaffected (Fig. 4H, lane 2-4). Histological examination revealed the presence of dorsal mesoderm such as notochord and muscle. The ventral mesodermal tissues such as coelomic epidermis and blood-like cells could not be observed in BMP-4-treated dorsal lips (Table 2). In addition, the elongation of notochord was observed following treatment with high concentrations of BMP-4 (data not shown). Dorsal lips are therefore affected qualitatively by BMP-4-treatment without changing their differentiating ability.

Table 2. Differentiation of dorsal lip explants treated with BMP-4.

BMP-4 (ng/ml, 3h)	0	10	100
No. of specimens	12	5	9
notochord	12(100)	5(100)	9(100)
muscle	9(75)	4(80)	9(100)
neural tissues	9(75)	1(20)	2(22)
epidermis	3(25)	0(0)	0(0)
mesenchyme	7(58)	2(40)	8(89)
atypical epidermis	0(0)	0(0)	0(0)
endodermal masses	10(83)	3(60)	9(100)

Figure in parentheses indicate the percentage of the number of specimens.

BMP-4 can also change the inducing ability of explants treated with a high concentration of activin.

To confirm that BMP-4 indeed has a posteriorizing effect on dorsal lips from stage 10.25 embryos, the more simple animal cap assay was used. This assay can exclude

the possibility that the observed BMP-4 effects were not due to another element of the dorsal lip region. In 1994, Ariizumi and Asashima showed that ectodermal explants treated with a high concentration of activin and precultured for a long time induced further anterior tissues in adjacent ectodermal explants (Ariizumi and Ashasima, 1994). Initially, we investigated whether the head-inducing ability of explants treated with activin was changed by BMP-treatment, as seen with

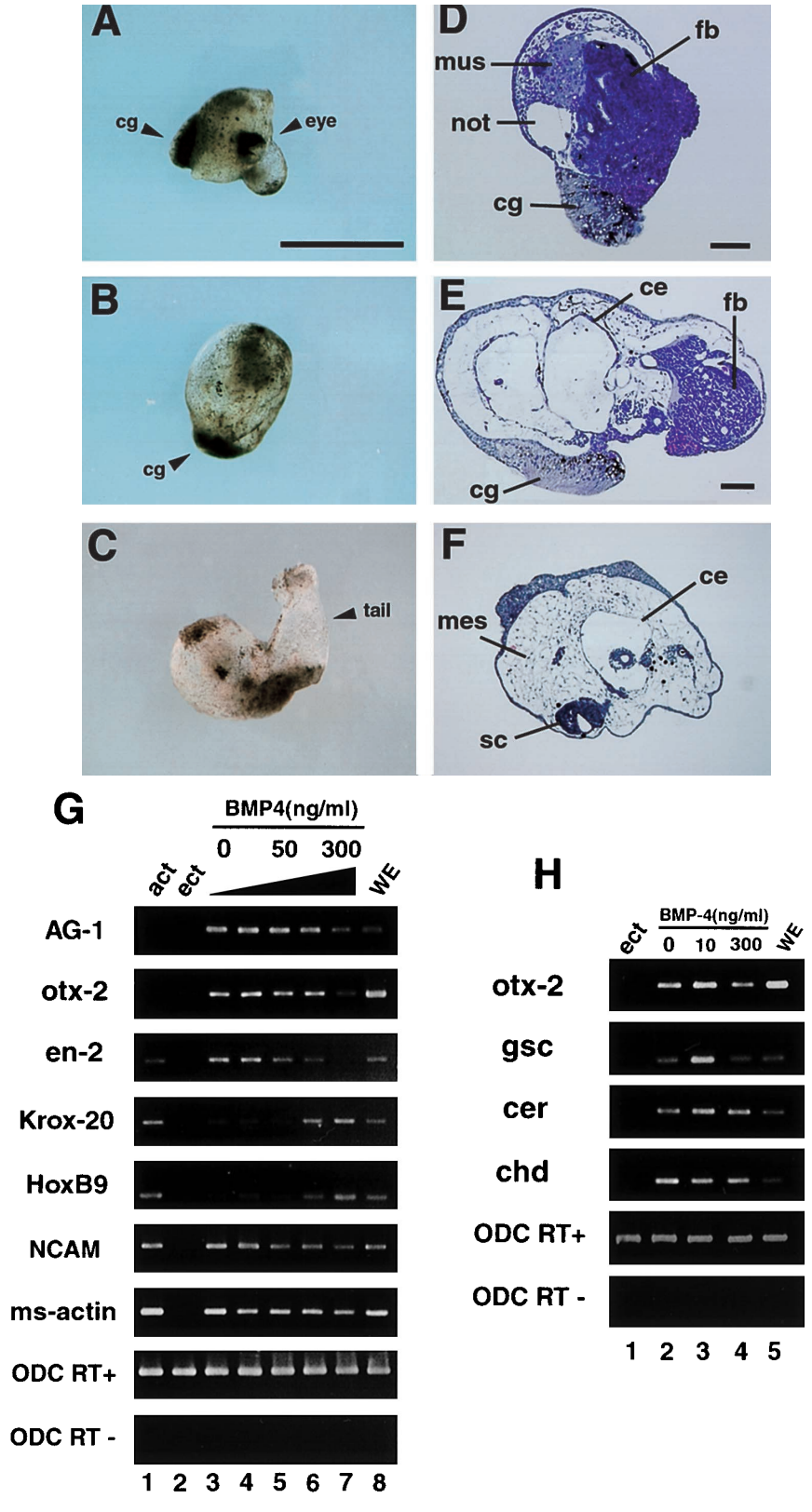


Fig. 5. BMP-4 changed the inducing ability of activin-treated explants.
External view (A-C) and histological section (D-F) of the explants at stage 42. Sandwich-explants of activin-treated (A, D), or BMP-4 treated at 10 ng/ml (B, E), or 100 ng/ml (C, F). The activin-treated explants differentiated into anterior structures. The cement gland and forebrain were observed (A, D). At the middle of the BMP-4 concentration range, both anterior and trunk-tail structures were observed (B, E). At high concentrations, trunk-tail structures were observed (C, F). Note that BMP-4 changed the inducing ability of activin-treated explants from anterior to posterior, as it did for the dorsal lips. Scale bar represents 1 mm (A) or 100 μm (D,E).
(G) RT-PCR analysis was performed on sandwich explants cultured until sibling embryos reached stage 20. At low concentrations of BMP-4, the anterior neural markers, *otx-2* and *en-2* were expressed (lane 3, 4). In contrast, at high BMP-4 concentrations, the posterior neural markers, *Krox-20* and *HoxB9* were expressed (lane 6, 7). Anterior neural markers, *otx-2* and *en-2* were attenuated (lane 7). The activin-treated explants cultured alone expressed *Krox-20* and *HoxB9* (posterior neural markers), *NCAM* (pan-neural marker), and *ms-actin* (lane 1).
(H) RT-PCR analysis was performed on activin-treated explants cultured alone until sibling embryos reached stage 12.5. At high concentrations of BMP-4, *otx-2* and *gsc* were suppressed (lane 4). Other markers were unaffected (lane 2-4). Non-treated ectodermal explants expressed any marker genes (lane 1).

the dorsal lips. The presumptive ectoderm explanted at stage 9 was treated with 300 ng/ml of activin for 1 hr and precultured for 3 hr. The concentration of activin was determined based on results showing that activin-treated explants are constantly differentiated into both endodermal masses and axial mesoderm such as muscle and notochord (data not shown). After preculturing, activin-treated explants were treated with BMP-4 (Fig. 1C, see Materials and Methods). To monitor tissue differentiation, the sandwich-explants were cultured until sibling embryos reached stage 42 (Fig. 5A–F). The activin-treated explants without BMP-4-treatment induced anterior structures (Fig. 5A, D), with cement gland, eye, and forebrain being observed (Fig. 5D). These data indicate that activin-treated explants precultured for 3 hrs mimicked the head organizer activity. In the middle of the BMP-4 concentration range, activin-treated explants induced both anterior and trunk-tail structures in adjacent ectoderm (Fig. 5B, E). At high concentrations, trunk-tail structures were induced (Fig. 5C, F). The coelomic epidermis, mesenchyme, spinal cord, and muscle were observed in histology (Fig. 5F). RT-PCR analysis detected expression of the anterior neural markers, *otx-2* and *en-2* at low BMP-4 concentrations (Fig. 5G, lane 3, 4, 5). At high concentrations, the posterior neural markers, *Krox-20* and *HoxB9* were upregulated (Fig. 5G, lane 6, 7) and anterior neural markers, *otx-2* and *en-2* were attenuated (Fig. 5G, lane 7). The activin-treated explants cultured alone expressed posterior markers, *Krox-20* and *HoxB9* (Fig. 5G, lane 1). Non-treated sandwich explants expressed any marker gene tested (Fig. 5G, lane 2). This result suggests that activin-treated explants lose head inducing ability and induce trunk-tail structures following BMP-4-treatment in a dose dependent manner (Table 3).

Simultaneously, we performed the isolation culture of activin-treated explants that were similarly treated with BMP-4 to detect the expression of organizer marker genes or to observe morphological changes. RT-PCR analysis showed that the head organizer markers, *otx-2* and *gsc* were suppressed by high concentration of BMP-4 (Fig. 5H, lane 4). The transcriptional levels of neural inducer *chd* and another head organizer marker *cer* were unaffected (Fig. 5H, lane 2-4). Other markers, neural inducer *nog*, pan-mesodermal marker *Xbra*, and notochord marker *Xnot*, were also unaffected (data not shown). Non-treated ectodermal explants expressed any marker gene (Fig. 5H, lane 1). Histological examination identified dorsal mesoderm tissues such as muscle and notochord in the activin-treated explants treated with BMP-4 (Table 4). There was no ventral mesoderm observed, such as blood-like cells and coelomic epidermis. These assay results indicated that activin-treated explants were affected qualitatively by BMP-4-treatment without changing their differentiating ability. The elongation of notochord observed in the dorsal lips was not observed in activin-treated explants. This difference may result from a variation in the competence for BMP-4 between dorsal lip and activin-treated explants.

Table 3. Differentiation of sandwich explants treated with activin and BMP-4.

	BMP-4 (ng/ml, 3h)	0	10	50	100
	No. of specimens	19	28	20	29
head	cement gland	17(89)	21(75)	4(20)	12(41)
	forebrain	15(79)	14(50)	1(5)	5(17)
	eye	7(37)	6(21)	1(5)	1(3)
trunk	hindbrain	1(5)	9(32)	1(5)	3(10)
tail	spinal cord	1(5)	0(0)	0(0)	13(45)
	mesenchyme	13(68)	23(82)	19(95)	27(93)
	coelomic epidermis	5(26)	12(43)	14(70)	20(69)
	epidermis	18(95)	26(93)	16(80)	20(69)
	notochord	1(5)	3(11)	10(50)	4(14)
	muscle	17(89)	21(75)	9(45)	15(52)
	endodermal masses	5(26)	8(29)	1(5)	3(10)
	atypical epidermis	1(5)	4(14)	6(30)	9(31)

Figure in parentheses indicate the percentage of the number of specimens.

Table 4. Differentiation of activin-treated explants treated with BMP-4.

BMP-4 (ng/ml, 3h)	0	10	300
No. of specimens	14	16	11
notochord	5(36)	2(13)	3(27)
muscle	14(100)	16(100)	11(100)
neural tissues	12(86)	11(69)	7(64)
epidermis	12(86)	7(44)	9(82)
mesenchyme	9(64)	3(19)	4(36)
atypical epidermis	0(0)	0(0)	0(0)
endodermal masses	7(50)	16(100)	7(73)

Figure in parentheses indicate the percentage of the number of specimens.

BMP-4 directly affects the head inducing ability of dorsal mesoderm via an intercellular signaling pathway.

The question remains as to how BMP-4 actually modulates the inducing ability of dorsal lips and activin-treated explants. There are two possibilities; one is by direct interaction with a neural inducer, such as Noggin, Chordin, or Follistatin, and another is via a signaling pathway. To address this, we examined the effect of BMP-4 in tissues injected with tBR mRNA to block the BMP-4 signaling pathway, as described in Materials and Methods. To avoid the possibility that head structures may be induced by tBR injection, tBR injected ectodermal explants were sandwiched between non-treated ectodermal explants (Fig. 1D, see Materials and Methods).The external view of the explants cultured to stage 42 is indicated in Fig. 6A-D. The activin-treated explants injected with tBR were able to induce head structures following BMP-4-treatment (Fig. 6C), whereas uninjected explants induced trunk-tail structures (Fig. 6D). tBR mRNA injected ectodermal explants sandwiched between ectodermal explants differentiated into atypical epi-

dermis (Fig. 6A). The activin-treated explants (but not BMP-4-treated) injected with tBR mRNA induced head structures as seen in uninjected controls (Fig. 5A, 6B). RT-PCR analysis was performed at stage 20 to detect the expression of positional markers, and detected the expression of the anterior neural markers, *otx-2* and *en-2* following BMP-4-treatment in sandwich explants of activin-treated explants injected with tBR mRNA (Fig. 6E, lane 5). In contrast,

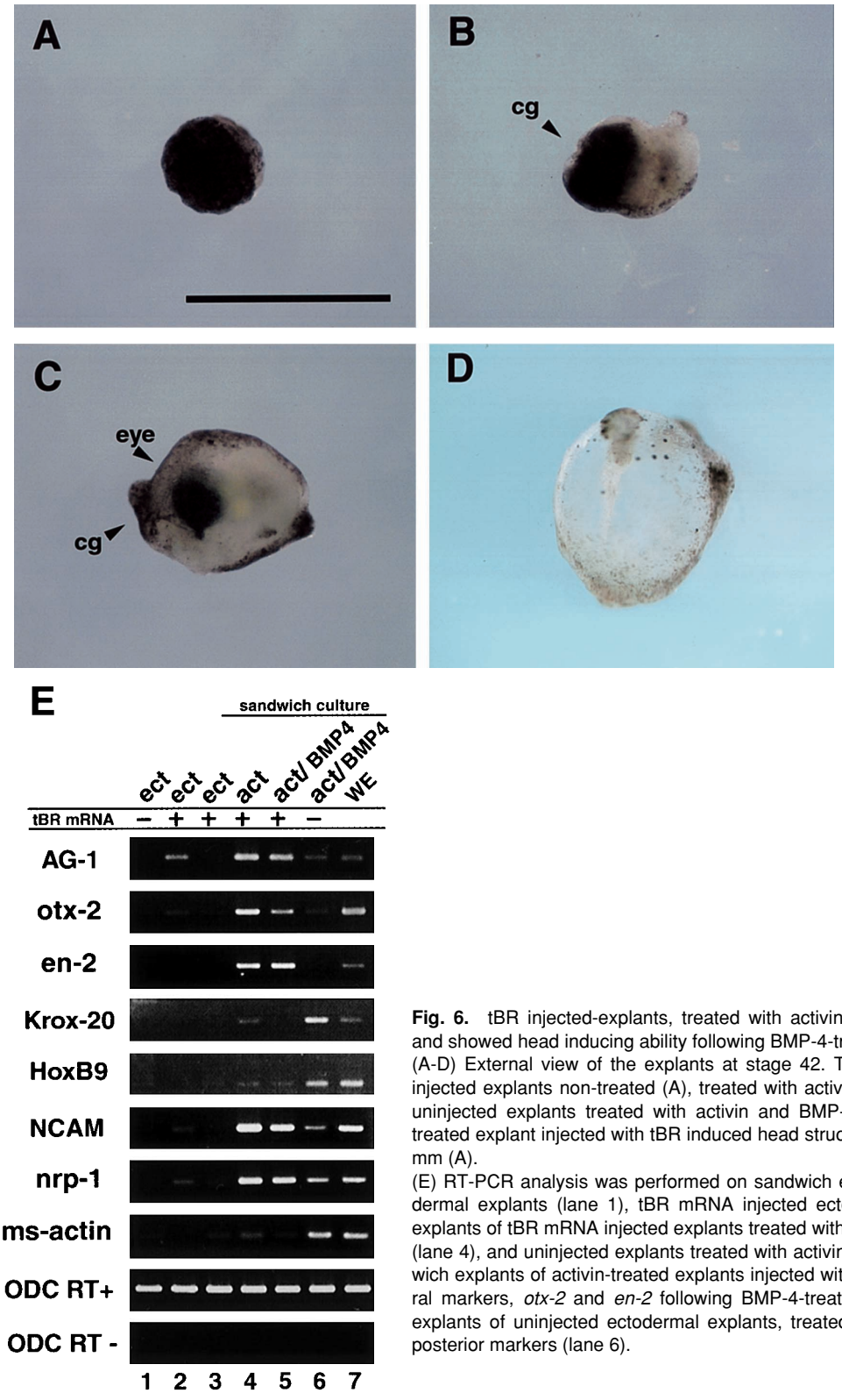


Fig. 6. tBR injected-explants, treated with activin, expressed head organizer markers and showed head inducing ability following BMP-4-treatment.
(A-D) External view of the explants at stage 42. The sandwich explants of tBR mRNA injected explants non-treated (A), treated with activin (B), or activin and BMP-4 (C), and uninjected explants treated with activin and BMP-4 (D). Note that activin and BMP-4 treated explant injected with tBR induced head structures (C). Scale bar in A represents 1 mm (A).
(E) RT-PCR analysis was performed on sandwich explants at stage 20; uninjected ectodermal explants (lane 1), tBR mRNA injected ectodermal explants (lane 2), sandwich explants of tBR mRNA injected explants treated with activin (lane 3), or activin and BMP-4 (lane 4), and uninjected explants treated with activin and BMP-4 (lane 5). Note that sandwich explants of activin-treated explants injected with tBR mRNA expressed anterior neural markers, *otx-2* and *en-2* following BMP-4-treatment (lane 5). In contrast, sandwich explants of uninjected ectodermal explants, treated with activin and BMP-4, expressed posterior markers (lane 6).

uninjected sandwich explants treated with activin and BMP-4 expressed posterior markers, *Krox-20* and *HoxB9* (Fig. 6E, lane 6). Uninjected ectodermal explants did not express any marker (Fig. 6E, lane 1). tBR mRNA injected explants cultured alone expressed *AG-1* and *otx-2,* as previously reported (Fig. 6E, lane 2) (Salzberg *et al*., 1999). In addition, tBR mRNA injected explants sandwiched between ectodermal explants did not express any marker gene (Fig. 6E, lane 3). In an isolation culture, activin-treated explants injected with tBR expressed the head organizer marker, *otx-2* and *gsc* following BMP-4-treatment (data not shown). These data suggest that BMP-4 affects the head inducing ability of activin-treated explants via an intercellular signaling pathway.

In summary, this study provided a number of clear results. The dorsal lips from stage 10.25 embryos can act as the head organizer after 6 hrs. Further to this, the dorsal lips conjugated with ectodermal explants containing VMZ cells differentiated into both anterior and posterior tissues along the A-P axis. Both dorsal lips from stage 10.25 embryos and activin-treated explants were found to have competence for BMP-4, and as a result of BMP-4-treatment, dorsal lips of early gastrulae and activin-treated explants induced trunk-tail structures. Finally, blocking of the BMP-4 signaling pathway in activin-treated explants abrogated the effect of BMP-4, implicating an indirect action for BMP-4 via signal transduction.

DISCUSSION

The results presented here show that the dorsal lips of stage 10.25 embryos had head inducing ability for a culturing period until sibling embryos reached stage 12, after which they induced trunk-tail structures. This indicates that the head inducing ability of the early dorsal lip region is unaffected by removal from the embryos and segregation from different kind of cells. Conversely, the dorsal lips from early gastrulae induced both head and trunk-tail structures when combined with ectodermal explant containing VMZ cells, implying that complete A-P axis patterning needs marginal zone cells (non-axial mesoderm) in addition to dorsal marginal zone cells (dorsal mesoderm). There are many reports that support the importance of marginal zone cells (non-axial lateral mesoderm) to the development of posterior structures, however no experiments have directly addressed A-P axis patterning in the absence of lateral mesoderm (Games and Sive, 2000). Our study directly implicates the importance of non-axial lateral mesoderm in posterior patterning.

It is interesting to speculate why does dorsal mesoderm induces posterior structures when combined with marginal zone cells. Two hypotheses can be proposed to account for this. One is that the anterior neural tissues, induced by dorsal mesoderm, are caudalized by marginal zone cells (lateral mesoderm) to invaginate from the mid gastrulae. The second is that lateral mesoderm directly converts the anterior inducing ability of dorsal mesoderm to induce posterior structures. In this study, we investigated the second hypothesis. BMP-4 is a candidate as one of caudalizing factors of marginal zone cells. Our data indicate that BMP-4 confers trunk-tail inducing ability to dorsal lip and activin-treated explants in a dose dependent manner. In addition, tBR-injection inhibited the caudalizing effect of BMP-4. Taken together this implies that BMP-4 acts to influence the head inducing ability of dorsal mesoderm directly through its intercellular signaling pathway. BMP-4 may play an important role in patterning of the A-P axis. BMP-4 is known to combine with its receptor and activate or attenuate the expression of target genes. In this way, BMP-4 may inhibit the expression of an anterior inducer, which gives positional information to neighboring cells or maintains the nature of the anterior region by excluding posteriorizing factors, and initiate posterior induction by upregulation of a posterior inducer. Recent studies support this prediction (Jones *et al*., 1992, 1996; Fainsod *et al*., 1994; Schmidt *et al*., 1995, 1996). The posterior structures of embryos dorsalized by treatment with LiCl were rescued by BMP-4 (Fainsod *et al*., 1994). In addition, expression of posterior-markers such as *Xhox*-3 and *pintallavis* was markedly increased in embryos injected with BMP-4 mRNA, although the expression of organizer-specific genes such as *goosecoid*, *Xnot*, and *noggin* were downregulated as gastrulation proceeded (Jones *et al*., 1996).

In our study, dorsal lips and activin-treated explants were affected qualitatively by BMP-4 without altering their differentiating ability. The expression of head organizer markers, *otx-2* and *gsc* were suppressed by BMP-4 treatment, however, the transcriptional level of *cer* was unaffected. The marker *cer* is regulated by other factors such as *Xwnt*. In normal development, *otx-2* and *gsc* are expressed in the anterior region as gastrulation proceeds. The expression of *otx-2* and *gsc* in the posterior region of late gastrulae may be suppressed by factors released from invaginating non-axial mesoderm. On the other hand, the expression of the neural inducer, *chd* and *nog* were unaffected. This result suggests the possibility that the factors defining a region may be important to the patterning of the A-P axis. Initially, pan neural inducers induce neural cells. As gastrulation proceeds, dorsal cells invaginate toward the anterior region and then VMZ cells invaginate into the embryo by convergence and extension (Keller, 1991). Following gastrulation, the factors defining the specific regions give positional information of the A-P axis to neuralized cells. As a result, neuralized cells that received this information differentiate into anterior structures such as cement gland and forebrain. In contrast, neuralized cells, which cannot receive anterior positional information or receive posterior positional information, differentiate into posterior structures such as hindbrain and spinal cord.

This study demonstrates the importance of BMP-4 to the neural inducing ability of dorsal mesoderm (dorsal lip region) in *Xenopus* gastrulae using an *in vitro* assay. BMP-

4 is able to confer trunk-tail inducing ability of dorsal mesoderm that normally act as the head inducer. The endogenous patterning of BMP and activin-like signals remains unclear. Further investigation of the locational expression of BMP-4 would contribute to our understanding of the mechanisms that determine body axes development in vertebrates.

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